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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/82, 9/02, 15/53, 5/04, A01H 5/00, 5/10 — A2

(11) International Publication Number: WO 99/50430

(43) International Publication Date: 7 October 1999 (07.10.99)

(21) International Application Number: PCT/US99/06765

(22) International Filing Date: 29 March 1999 (29.03.99)

(30) Priority Data:
60/079,840 30 March 1998 (30.03.98) US

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(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published

Without international search report and to be republished upon receipt of that report.

(54) Title: MODIFICATION OF FATTY ACID COMPOSITION IN PLANTS BY EXPRESSION OF AN *ASPERGILLUS NIDULANS* DELTA-9 COA DESATURASE

(57) Abstract

Genes encoding a palmitate Δ–9 desaturase from *Aspergillus nidulans* have been isolated. The proteins encoded by said genes, when expressed in a plant, can alter the saturate levels of the oil.

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MODIFICATION OF FATTY ACID COMPOSITION IN PLANTS BY EXPRESSION OF AN *ASPERGILLUS NIDULANS* DELTA-9 COA DESATURASE

Cross-reference to Related Application

This patent application claims priority from a U.S. Provisional Patent Application Serial Number 60/079840, filed March 30, 1998.

Field of Invention

This invention relates to the preparation and use of nucleic acid fragments or genes which encode fungal palmitoyl-CoA Δ-9 desaturase enzymes to create transgenic plants having altered oil profiles.

Background of the Invention

Plant-produced oils can be found in a wide variety of products including lubricants and foods. Interestingly, different plant species synthesize various oil types. For example, coconut and palm plants produce oils that are abundant in fatty acids having medium chain lengths (10-12 carbon atoms). These oils are used in manufacturing soaps, detergents and surfactants, and represent a U.S. market size greater than $350 million per year. Other plants, such as rape, produce oils abundant in long chain fatty acids (22 carbon atoms) and are used as lubricants and anti-slip agents. Additional applications of plant oils include their use in plasticizers, coatings, paints, varnishes and cosmetics (Volker et al., (1992) Science 257:72-74; Ohlrogge, (1994) Plant Physiol. 104:821-826). However, the predominant use of plant oils is in the production of food and food products.

Over the years, vegetable-derived oils have gradually replaced animal-derived oils and fats as the major source of dietary fat intake. However, saturated fat intake in most industrialized nations has remained at about 15% to 20% of total caloric consumption. In efforts to promote

healthier lifestyles, the United States Department of Agriculture(USDA) has recently recommended that saturated fats make up less than 10% of daily caloric intake. To facilitate consumer awareness, current labeling guidelines issued by the USDA now require total saturated fatty acid levels be less than 1.0 g per 14 g serving to receive the "low-sat" label and less than 0.5 g per 14 g serving to receive the "no-sat" label. This means that the saturated fatty acid content of plant oils needs to be less than 7% and 1.75% to receive the "low sat" and "no sat" label, respectively. Since issuance of these guidelines, there has been a surge in consumer demand for "low-sat" oils. To date, this has been met principally with canola oil, and to a much lesser degree with sunflower and safflower oils.

The characteristics of oils, whether of plant or animal origin, are determined predominately by the number of carbon and hydrogen atoms, as well as the number and position of double bonds comprising the fatty acid chain. Most oils derived from plants are composed of varying amounts of palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2) and linolenic (18:3) fatty acids. Conventionally, palmitic and stearic acids are designated as "saturated" since the fatty acid chains have 16 and 18 carbon atoms, respectively, and no double bonds. They therefore contain the maximal number of hydrogen atoms possible. However, oleic, linoleic, and linolenic are 18-carbon fatty acid chains having one, two, and three double bonds, respectively, therein. Oleic acid is typically considered a mono-unsaturated fatty acid, whereas linoleic and linolenic are considered to be poly-unsaturated fatty acids.

Saturated fatty acids are linear molecules and tend to form self-stacked structures thereby resulting in high melting temperatures; a characteristic that is quite desirable when producing foods like chocolate. Animal fats, which are also solid at room temperature, are another readily available source of saturated fatty acids. However, use of said oil is often discouraged due to the high levels of cholesterol associated therewith. In comparison, unsaturated fatty acid chains are nonlinear due to bending induced by double bond insertion. The bending of the molecule impedes the ability of the fatty acid chains to stack thus causing them to remain fluid at lower temperatures. Vegetable oils, for example, are high in unsaturated fatty acids, and therefore are typically liquid at room temperature. Furthermore, saturated fatty acid can be modified to become unsaturated fatty acids by removal of hydrogen atoms and insertion of double bonds between two carbon atoms on the fatty acid chain. Desaturation can be achieved either enzymatically or chemically and decreases melting points due to the inability of the fatty acid molecules to self-stack.

The total saturated fatty acid level of corn oil, averaging about 13.9%, does not meet the current labeling guidelines discussed above. On average, corn oil is comprised of 11.5% palmitic acid, 2.2% stearic acid, 26.6% oleic acid, 58.7% linoleic acid, and 0.8% linolenic acid. Corn oil also contains 0.2% arachidic acid, a twenty-carbon saturated fatty acid (Dunlap et. al., (1995) J. Amer. Oil Chem. Soc. 72:981-987). The composition of corn oil instills it with properties that are most desirable in edible oils. These include properties such as heat stability, flavor, and long shelf life. However, consumer demand for "low sat" oils has resulted in a

significant decrease in corn oil utilization and thus decreased market share. Therefore, a corn oil with modified levels of saturated fatty acids is highly desirable and would have practical use in that it would meet the consumer demand for healthier oils while having most or all of the properties that made corn oil a popular and preferred oil in the past.

Corn is typically not considered to be an oil crop as compared to soybean, canola, sunflower and the like. In fact, the oil produced and extracted from corn is considered to be a byproduct of the wet milling process used in starch extraction. Because of this, there has been little interest in modifying the saturate levels of corn oil until those efforts disclosed herein.

As disclosed herein, saturate levels of fatty acids comprising plant oils can be altered by expressing a fungal palmitate-CoA Δ-9 desaturase within a plant cell. These proteins most likely enzymatically desaturate palmitate-CoA molecules by removing two hydrogen atoms and adding a double bond between the 9^{th} and 10^{th} carbon atoms from the CoA portion of the molecule, thus producing palmitoleic-CoA ($16:1^{\Delta 9}$). The palmitoleic-CoA is ultimately incorporated into seed oil thus lowering the total saturate levels of said oil.

Summary of the Invention

In the present invention, a gene encoding a fungal palmitate-CoA Δ-9 desaturase has been isolated and cloned from *Aspergillus nidulans*. The saturate level of oils found in plant cells can be altered by expressing said palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans*.

One aspect of the disclosed invention is a gene encoding said palmitate-CoA Δ-9 desaturase, said gene being isolated and purified from *Aspergillus nidulans*.

An additional aspect of the present invention relates to producing a gene wherein the codon bias of a gene from a non-plant source has been modified to look similar to genes from a plant source.

Another aspect of the invention relates to altering oil saturate levels within a plant cell by expressing said genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans*. Genes disclosed herein can be used to alter saturate levels by placing said genes in the sense orientation. Plants cells being transformed with genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* in the sense orientation results in the oils of said plants having increased 16:1 levels and decreased total saturate levels over non-transformed plants.

An additional aspect of the present invention is the production of chimeric genes using the genes disclosed herein encoding for palmitoyl CoA-Δ-9 desaturase in combination with promoter regulatory elements and the use of said chimeric genes within a plant cell.

Yet an additional aspect of the present invention is the transformation of plant species disclosed herein with said chimeric genes.

Other aspects, embodiments, advantages, and features of the present invention will become apparent from the following specification.

Detailed Description of the Invention

The present invention relates to methods and compositions for obtaining transgenic plants wherein plant

oils produced thereby have altered saturate levels. The following phrases and terms are defined below:

By "altered saturate levels" is meant that the level of total saturated fatty acids of a plant oil produced by a modified plant is different from that of a normal or non-modified plant.

By "cDNA" is meant DNA that is complementary to and derived from a mRNA.

By "chimeric DNA construction" is meant a recombinant DNA containing genes or portions thereof from one or more species.

By "complementarity" is meant a nucleic acid that can form hydrogen bond(s) with other nucleic acid sequences either through traditional Watson-Crick or other non-traditional types of base paired interactions.

By "constitutive promoter" is meant promoter elements that direct continuous gene expression in all cell types and at all times (i.e., actin, ubiquitin, CaMV 35S, 35T, and the like).

By "developmental specific" promoter is meant promoter elements responsible for gene expression at specific plant developmental stages, such as in early or late embryogenesis and the like.

By "enhancer" is meant nucleotide sequence elements which can stimulate promoter activity such as those from maize streak virus (MSV) protein leader sequence, alfalfa mosaic virus protein leader sequence, alcohol dehydrogenase intron 1, and the like.

By "expression" as used herein, is meant the transcription and stable accumulation of mRNA inside a plant cell. Expression of genes also involves transcription of the gene to create mRNA and translation of the mRNA into precursor or mature proteins.

By "foreign" or "heterologous gene" is meant a gene encoding a protein whose exact amino acid sequence is not normally found in the host cell, but is introduced by standard gene transfer techniques.

By "gene" is meant to include all genetic material involved in protein expression including chimeric DNA constructions, genes, plant genes and portions thereof, and the like.

By "genome" is meant genetic material contained in each cell of an organism and/or virus and the like.

By "inducible promoter" is meant promoter elements which are responsible for expression of genes in response to a specific signal such as: physical stimuli (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites, chemicals, stress and the like.

By "modified plant" is meant a plant wherein the gene, mRNA, or protein from *Aspergillus nidulans* palmitate-CoA Δ-9 desaturase is present.

By "plant" is meant a photosynthetic organism including both eukaryotes and prokaryotes.

By "promoter regulatory element" is meant nucleotide sequence elements within a nucleic fragment or gene which controls the expression of that nucleic acid fragment or gene. Promoter sequences provide the recognition for RNA polymerase and other transcriptional factors required for efficient transcription. Promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express gene constructs. Promoter regulatory elements are also meant to include constitutive, tissue-specific, developmental-specific, inducible promoters and the like. Promoter regulatory elements may also include certain enhancer sequence elements and the like that improve transcriptional efficiency.

By "tissue-specific" promoter is meant promoter elements responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (i.e., zein, oleosin, napin, ACP, globulin and the like).

By "transgenic plant" is meant a plant expressing a chimeric gene introduced through transformation efforts.

In plant cells, fatty acids are made as acyl-acyl carrier protein (acyl-ACP) substrates and are elongated by various enzymes through the addition of malonyl-ACP to make acyl-ACP molecules ranging in length from 2 to 18 carbon atoms. Afterwards, acyl-ACP thioesterases catalyze the hydrolytic cleavage of palmitic acid, stearic acid, and oleic acid from ACP, in a somewhat selective although not specific manner, thus producing a free fatty acids. The fatty acid molecules move out of the plastid into the cytoplasm where they are eventually modified into acyl-CoA molecules. Said molecules are then incorporated onto the triglyceride oil fraction. It has been discovered by applicants as disclosed herein that desaturation of an acyl-CoA molecule, wherein said molecule is preferably stearoyl-CoA and most preferably palmitate-CoA, can reduce saturate levels in the triglyceride oil fraction. Said desaturation most preferably results in the production and accumulation of palmitoleic acid ($16:1^{\Delta-9}$). Said desaturation may also result in a decrease in palmitic and stearic acid in the triglyceride oil fraction.

In corn seed oil, the predominant fatty acids are linoleic acid (18:2 at about 59%), oleic acid (18:1 at about 26%) and palmitic (16:0 at about 11%), with stearic acid (18:0) generally comprising about 2.5% or less (Glover and Mertz, (1987) in: Nutritional Quality of Cereal Grains: genetic and agronomic improvement., p.183-

336, (eds. Olson, R.A. and Frey, K.J.) Amer. Soc. Agronomy, Inc., Madison, WI; Fitch-Haumann, (1985) J. Am. Oil. Chem. Soc. 62:1524-1531). Biosynthesis of fatty acids in plant cells is initiated in the plastids where they are synthesized as acyl-ACP thioesters by a fatty acid synthase complex. More specifically, fatty acid production is accomplished by a series of condensation reactions involving addition of malonyl-ACP sequentially to a growing fatty acid-ACP chain by the enzyme β-ketoacyl-ACP synthase I (KAS I). Most fatty acid-ACP units reach carbon chain lengths of 16 and are then elongated to 18 carbon units by KAS II. The vast majority of C18 fatty acids become desaturated by stearoyl-ACP Δ-9 desaturase at the C9 position from the carboxyl end to produce oleyl-ACP.

Both saturated and unsaturated fatty acid-ACP units are hydrolyzed by acyl-ACP thioesterases to produce free fatty acids. These free fatty acids then cross the plastid membrane to the cytosol of the cell where they are modified by addition of a CoA moiety. Afterwards, said fatty acids are incorporated into plant oils (Somerville and Browse, (1991) Science 252:80-87; Browse and Sommerville (1991) Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:467-506; Harwood (1989) Critical Reviews in Plant Sci. 8:1-43; Chasan (1995) Plant Cell 7:235-237; Ohlrogge (1994) Plant Physiol. 104:821-826).

The palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* desaturates palmitic acid at the C9 position relative to the carboxyl end most likely after the point of modification with Co-A. In plant cells, this most likely occurs before being incorporated into the triglyceride fraction of the oil. Therefore, expressing

palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* in plant cells will cause a decrease in the saturate levels of the oil produced by said plant.

The palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* disclosed herein can be used to modify saturate levels in oil in both monocotyledonous and dicotyledonous plants. In dicotyledonous plants, expression of said desaturase preferably results in a decrease in 16:0 and 18:0 levels found in oil derived from said plants. More preferably, expression of said desaturase results in increased levels of 16:1 fatty acid in said oil. In monocotyledonous plants, expression of said desaturase preferably results in decreased levels of 18:0 and more preferably, increased levels of 16:1 found in the said oil. It is not applicants intention, however, to limit said gene expression exclusively to plants in that said desaturase and genes thereof can be expressed and used to modify lipid contents in both yeast and bacteria.

As further described herein, an *Aspergillus* palmitate-CoA Δ-9 desaturase can be used to modify the saturate levels in oils produced by transgenic plants. Preferably, genes and nucleic fragments encoding the palmitate-CoA Δ-9 desaturase are derived from *Aspergillus nidulans*. More preferably, genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* are those disclosed herein as SEQ ID NO:5 and SEQ ID NO:12, said genes encoding a protein having an amino acid sequence as disclosed herein as SEQ ID NO:6.

One method by which plant oils can be modified is by expressing the palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* in a dicotyledonous plant. This can be achieved by placing the genes or nucleic acid fragments

encoding said proteins in the sense orientation 3' to a promoter regulatory element of choice followed by a transcriptional terminator at the 3' end of said gene thus producing a chimeric gene construct. These chimeric genes can then be transformed into plants, thereby producing plant oils having altered saturate levels relative to nontransformed controls. Expressing the palmitate-CoA Δ-9 desaturase as disclosed herein from *Aspergillus nidulans* in dicotyledonous plants results in plant oils derived therefrom having 16:1 levels as a percentage of the total fatty acid from about 0.23 to about 4.65%; preferably from about 3.01 to about 4.65%; more preferably from about 4.07 to about 4.65%, with about 4.65% being most preferred. The total saturate levels range preferably from about 9.8 to about 12.5% with about 9.8% being most preferred.

Another method by which plant oils can be modified is by expressing the palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* in a monocotyledonous plant. As with dicotyledonous plants, this can be achieved by placing the genes or nucleic acid fragments encoding said proteins in the sense orientation 3' to a promoter regulatory element of choice followed by a transcriptional terminator at the 3'end of said gene thus producing a chimeric gene construct. These chimeric genes can then be transformed into plants, thereby producing plant oils having altered saturate levels relative to nontransformed controls. Expressing the palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* in monocotyledonous plants results in plant oils derived therefrom to have 16:1 levels from about 0.4 to about 3.2%; preferably from about 1.2 to about 3.2%, with about 3.2% being most preferred.

As further disclosed herein, chimeric gene constructs encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* can be transformed in other oilseed crops to modify the saturate levels therein. Said oilseed crop plant species which may be modified include but are not limited to soybean, *Brassicaceae* sp., canola, rape, sunflower, flax, safflower, coconut, palm, olive, peanut, cotton, castor bean, coriander, *Crambe* sp., *Cuphea* sp., *Euphorbia* sp., *Oenothera* sp., jojoba, *Lesquerella* sp., marigold, *Limnanthes* sp., *Vernonia* sp., *Sinapis alba*, and cocoa, with maize being most preferred. Most if not all of these plant species have been previously transformed by those having ordinary skill in the art.

To obtain high expression of heterologous genes in plants it may be preferred to reengineer said genes so that they are more efficiently expressed in the cytoplasm of plant cells. Maize is one such plant where it may be preferred to reengineer the heterologous gene(s) prior to transformation to increase the expression level thereof in said plant. Therefore, an additional step in the design of genes encoding said palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* is the designed reengineering of a heterologous gene for optimal expression.

One reason for the reengineering the Δ-9 Co-A desaturase gene from *Aspergillus nidulans* for expression in maize is due to the non-optimal G+C content of the native gene. For example, the very low G+C content of many native bacterial gene(s) (and consequent skewing towards high A+T content) results in the generation of sequences mimicking or duplicating plant gene control sequences that are known to be highly A+T rich. The presence of some A+T-rich sequences within the DNA of gene(s) introduced into plants (e.g., TATA box regions

normally found in gene promoters) may result in aberrant transcription of the gene(s). On the other hand, the presence of other regulatory sequences residing in the transcribed mRNA (e.g., polyadenylation signal sequences (AAUAAA), or sequences complementary to small nuclear RNAs involved in pre-mRNA splicing) may lead to RNA instability. Therefore, one goal in the design of genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* for maize expression, more preferably referred to as plant optimized gene(s), is to generate a DNA sequence having a higher G+C content, and preferably one close to that of maize genes coding for metabolic enzymes. Another goal in the design of the plant optimized gene(s) encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* is to generate a DNA sequence in which the sequence modifications do not hinder translation.

The table below (Table 1) illustrates how high the G+C content is in maize. For the data in Table 1, coding regions of the genes were extracted from GenBank (Release 71) entries, and base compositions were calculated using the MacVector™ program (IBI, New Haven, CT). Intron sequences were ignored in the calculations.

Due to the plasticity afforded by the redundancy of the genetic code (i.e., some amino acids are specified by more than one codon), evolution of the genomes in different organisms or classes of organisms has resulted in differential usage of redundant codons. This "codon bias" is reflected in the mean base composition of protein coding regions. For example, organisms with relatively low G+C contents utilize codons having A or T in the third position of redundant codons,

whereas those having higher G+C contents utilize codons having

Table 1. Compilation of G+C contents of protein coding regions of maize genes.

Protein Class[a]	Range %G+C	Mean %G+C[b]
Metabolic Enzymes (76)	44.4-75.3	59.0 (± 8.0)
Structural Proteins (18)	48.6-70.5	63.6 (± 6.7)
Regulatory Proteins (5)	57.2-68.9	62.0 (± 4.9)
Uncharacterized Proteins (9)	41.5-70.3	64.3 (± 7.2)
All Proteins (108)	**44.4-75.3**	**60.8 (± 5.2)**

[a]Number of genes in class given in parentheses.
[b]Standard deviations given in parentheses.
[c]Combined groups mean ignored in mean calculation.

G or C in the third position. It is thought that the presence of "minor" codons within a mRNA may reduce the absolute translation rate of that mRNA, especially when the relative abundance of the charged tRNA corresponding to the minor codon is low. An extension of this is that the diminution of translation rate by individual minor codons would be at least additive for multiple minor codons. Therefore, mRNAs having high relative contents of minor codons would have correspondingly low translation rates. This rate would be reflected by subsequent low levels of the encoded protein.

In reengineering genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* for maize expression, the codon bias of the plant has been determined. The codon bias for maize is the statistical codon distribution that the plant uses for coding its proteins and the preferred codon usage is shown in Table 2. After determining the bias, the percent frequency of the codons in the gene(s) of interest is determined. The primary

codons preferred by the plant should be determined as well as the second and third choice of preferred codons. Afterwards, the amino acid sequence of palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* is reverse translated so that the resulting nucleic acid sequence codes for exactly the same protein as the native gene wanting to be heterologously expressed. The new DNA sequence is designed using codon bias information so that it corresponds to the most preferred codons of the desired plant. The new sequence is then analyzed for restriction enzyme sites that might have been created by the modification. The identified sites are further modified by replacing the codons with second or third choice with preferred codons. Other sites in the sequence which could affect transcription or translation of the gene of interest are the exon:intron 5' or 3' junctions, poly A addition signals, or RNA polymerase termination signals. The sequence is further analyzed and modified to reduce the frequency of TA or GC doublets. In addition to the doublets, G or C sequence blocks that have more than about four residues that are the same can affect transcription of the sequence. Therefore, these blocks are also modified by replacing the codons of first or second choice, etc. with the next preferred codon of choice.

It is preferred that the plant optimized gene(s) encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* contain about 63% of first choice codons, between about 22% to about 37% second choice codons, and between about 15% to about 0% third choice codons, wherein the total percentage is 100%. Most preferred the plant optimized gene(s) contains about 63% of first choice codons, at least about 22% second choice codons, about 7.5% third choice codons, and about 7.5% fourth choice

codons, wherein the total percentage is 100%. The preferred codon usage for engineering genes for maize expression are shown in Table 2. The method described above enables one skilled in the art to modify gene(s) that are foreign to a particular plant so that the genes are optimally expressed in plants. The method is further illustrated in pending PCT application WO 97/13402, which is incorporated herein by reference.

In order to design plant optimized genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans*, the amino acid sequence of said protein is reverse translated into a DNA sequence utilizing a non-redundant genetic code established from a codon bias table compiled for the gene sequences for the particular plant, as shown in Table 2.

The resulting DNA sequence, which is completely homogeneous in codon usage, is further modified to establish a DNA sequence that, besides having a higher degree of codon diversity, also contains strategically placed restriction enzyme recognition sites, desirable base composition, and a lack of sequences that might interfere with transcription of the gene, or translation of the product mRNA. Said sequence produced using the methods described herein is disclosed as SEQ ID NO:12.

In another aspect of the invention, genes encoding the palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* are expressed from transcriptional units inserted into the plant genome. Preferably, said transcriptional units are recombinant vectors capable of stable integration into the plant genome and selection of transformed plant lines expressing mRNA encoding for said desaturase proteins are expressed either by constitutive or inducible promoters in the plant cell. Once expressed, the mRNA is translated

into proteins, thereby incorporating amino acids of interest into protein. The genes encoding palmitate-CoA Δ-9 desaturase from *Aspergillus nidulans* expressed in the plant cells can be under the control of a constitutive promoter, a tissue-specific promoter or an inducible promoter as described herein.

Table 2. Preferred amino acid codons for proteins expressed in maize.

Amino Acid	Codon*
Alanine	GCC/GCG
Cysteine	TGC/TGT
Aspartic Acid	GAC/GAT
Glutamic Acid	GAG/GAA
Phenylalanine	TTC/TTT
Glycine	GGC/GGG
Histidine	CAC/CAT
Isoleucine	ATC/ATT
Lysine	AAG/AAA
Leucine	CTG/CTC
Methionine	ATG
Asparagine	AAC/AAT
Proline	CCG/CCA
Glutamine	CAG/CAA
Arginine	AGG/CGC
Serine	AGC/TCC
Threonine	ACC/ACG
Valine	GTG/GTC
Tryptophan	TGG
Tryrosine	TAC/TAT
Stop	TGA/TAG
* The first and second preferred codons for maize.	

Several techniques exist for introducing foreign recombinant vectors into plant cells, and for obtaining plants that stably maintain and express the introduced gene. Such techniques include acceleration of genetic material coated onto microparticles directly into cells (U.S. Patents 4,945,050 to Cornell and 5,141,131 to DowElanco, now Dow AgroSciences). In addition, plants may be transformed using *Agrobacterium* technology, see U.S. Patent 5,177,010 to University of Toledo, 5,104,310 to Texas A&M, European Patent Application 0131624B1, European Patent Applications 120516, 159418B1 and 176,112 to Schilperoot, U.S. Patents 5,149,645, 5,469,976, 5,464,763 and 4,940,838 and 4,693,976 to Schilperoot, European Patent Applications 116718, 290799, 320500 all to Max Planck, European Patent Applications 604662,627752 and US Patent 5,591,616 to Japan Tobacco, European Patent Applications 0267159, and 0292435 and U.S. Patent 5,231,019 all to Ciba Geigy,now Novartis, U.S. Patents 5,463,174 and 4,762,785 both to Calgene, and U.S. Patents 5,004,863 and 5,159,135 both to Agracetus. Other transformation technology includes whiskers technology, see U.S. Patents 5,302,523 and 5,464,765 both to Zeneca. Electroporation technology has also been used to transform plants, see WO 87/06614 to Boyce Thompson Institute, 5,472,869 and 5,384,253 both to Dekalb, WO9209696 and WO9321335 both to Plant Genetic Systems. Furthermore, viral vectors can also be used in produce transgenic plants expressing the protein of interest. For example, monocotyledonous plant can be transformed with a viral vector using the methods described in U.S. Patents 5,569,597 to Mycogen and Ciba-Giegy, now Novartis, as well as U.S. Patents 5,589,367 and 5,316,931, both to

Biosource. All of these transformation patents and publications are incorporated herein by reference.

As mentioned previously, the manner in which the DNA construct is introduced into the plant host is not critical to this invention. Any method which provides for efficient transformation may be employed. For example, various methods for plant cell transformation are described herein and include the use of Ti or Ri-plasmids and the like to perform Agrobacterium mediated transformation. In many instances, it will be desirable to have the construct used for transformation bordered on one or both sides by T-DNA borders, more specifically the right border. This is particularly useful when the construct uses *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as a mode for transformation, although T-DNA borders may find use with other modes of transformation.

Where Agrobacterium is used for plant cell transformation, a vector may be used which may be introduced into the host for homologous recombination with T-DNA or the Ti or Ri plasmid present in the host. Introduction of the vector may be performed via electroporation, tri-parental mating and other techniques for transforming gram-negative bacteria which are known to those skilled in the art. The manner of vector transformation into the Agrobacterium host is not critical to this invention. The Ti or Ri plasmid containing the T-DNA for recombination may be capable or incapable of causing gall formation, and is not critical to said invention so long as the vir genes are present in said host.

In some cases where Agrobacterium is used for transformation, the expression construct being within the T-DNA borders will be inserted into the plasmid pDAB1542

as described herein or into a broad spectrum vector such as pRK2 or derivatives thereof as described in Ditta et al., (PNAS USA (1980) 77:7347-7351 and EPO 0 120 515, which are incorporated herein by reference. Included within the expression construct and the T-DNA will be one or more markers as described herein which allow for selection of transformed Agrobacterium and transformed plant cells. The particular marker employed is not essential to this invention, with the preferred marker depending on the host and construction used.

For transformation of plant cells using Agrobacterium, explants may be combined and incubated with the transformed Agrobacterium for sufficient time to allow transformation thereof. After transformation, the agrobacteria are killed by selection with the appropriate antibiotic and plant cells are cultured with the appropriate selective medium. Once calli are formed, shoot formation can be encourage by employing the appropriate plant hormones according to methods well known in the art of plant tissue culturing and plant regeneration. However, a callus intermediate stage is not always necessary. After shoot formation, said plant cells can be transferred to medium which encourages root formation thereby completing plant regeneration. The plants may then be grown to seed and said seed can be used to establish future generations as well as provide a source for oil isolation. Regardless of transformation technique, the gene encoding palmitoyl-CoA Δ-9 desaturase from *Aspergillus nidulans* is preferably incorporated into a gene transfer vector adapted to express said gene in a plant cell by including in the vector a plant promoter regulatory element, as well as 3' non-translated

transcriptional termination regions such as Nos and the like.

In addition to numerous technologies for transforming plants, the type of tissue which is contacted with the foreign genes may vary as well. Such tissue would include but would not be limited to embryogenic tissue, callus tissue types I, II, and III, hypocotyl, meristem, and the like. Almost all plant tissues may be transformed during dedifferentiation using appropriate techniques described herein.

Another variable is the choice of a selectable marker. Preference for a particular marker is at the discretion of the artisan, but any of the following selectable markers may be used along with any other gene not listed herein which could function as a selectable marker. Such selectable markers include but are not limited to aminoglycoside phosphotransferase gene of transposon Tn5 (Aph II) which encodes resistance to the antibiotics kanamycin, neomycin and G418, as well as those genes which encode for resistance or tolerance to glyphosate; hygromycin; methotrexate; phosphinothricin (bialophos); imidazolinones, sulfonylureas and triazolopyrimidine herbicides, such as chlorsulfuron; bromoxynil, dalapon and the like.

In addition to a selectable marker, it may be desirous to use a reporter gene. In some instances a reporter gene may be used with or without a selectable marker. Reporter genes are genes which are typically not present in the recipient organism or tissue and typically encode for proteins resulting in some phenotypic change or enzymatic property. Examples of such genes are provided in K. Weising et al. Ann. Rev. Genetics, 22, 421 (1988), which is incorporated herein by reference. Preferred

reporter genes include the beta-glucuronidase (GUS) of the uidA locus of *E. coli*, the chloramphenicol acetyl transferase gene from Tn9 of *E. coli*, the green fluorescent protein from the bioluminescent jellyfish *Aequorea victoria*, and the luciferase genes from firefly *Photinus pyralis*. An assay for detecting reporter gene expression may then be performed at a suitable time after said gene has been introduced into recipient cells. A preferred such assay entails the use of the gene encoding beta-glucuronidase (GUS) of the uidA locus of *E. coli* as described by Jefferson et al., (1987 Biochem. Soc. Trans. 15, 17-19) to identify transformed cells.

In addition to plant promoter regulatory elements, promoter regulatory elements from a variety of sources can be used efficiently in plant cells to express foreign genes. For example, promoter regulatory elements of bacterial origin, such as the octopine synthase promoter, the nopaline synthase promoter, the mannopine synthase promoter; promoters of viral origin, such as the cauliflower mosaic virus (35S and 19S), 35T (which is a re-engineered 35S promoter, see PCT/US96/1682; WO 97/13402 published April 17, 1997) and the like may be used. Plant promoter regulatory elements include but are not limited to ribulose-1,6-bisphosphate (RUBP) carboxylase small subunit (ssu), beta-conglycinin promoter, beta-phaseolin promoter, ADH promoter, heat-shock promoters and tissue specific promoters.

Other elements such as matrix attachment regions, scaffold attachment regions, introns, enhancers, polyadenylation sequences and the like may be present and thus may improve the transcription efficiency or DNA integration. Such elements may or may not be necessary for DNA function, although they can provide better

expression or functioning of the DNA by affecting transcription, mRNA stability, and the like. Such elements may be included in the DNA as desired to obtain optimal performance of the transformed DNA in the plant. Typical elements include but are not limited to Adh-intron 1, Adh-intron 6, the alfalfa mosaic virus coat protein leader sequence, the maize streak virus coat protein leader sequence, as well as others available to a skilled artisan.

Constitutive promoter regulatory elements may also be used thereby directing continuous gene expression in all cells types and at all times (e.g., actin, ubiquitin, CaMV 35S, and the like). Tissue specific promoter regulatory elements are responsible for gene expression in specific cell or tissue types, such as the leaves or seeds (e.g., zein, oleosin, napin, ACP, globulin and the like) and these may also be used.

Promoter regulatory elements may also be active during a certain stage of the plants' development as well as active in plant tissues and organs. Examples of such include but are not limited to pollen-specific, embryo specific, corn silk specific, cotton fiber specific, root specific, seed endosperm specific promoter regulatory elements and the like. Under certain circumstances it may be desirable to use an inducible promoter regulatory element, which is responsible for expression of genes in response to a specific signal, such as: physical stimulus (heat shock genes); light (RUBP carboxylase); hormone (Em); metabolites; chemical; and stress. Other desirable transcription and translation elements that function in plants may be used. Numerous plant-specific gene transfer vectors are known in the art.

One of the issues regarding exploiting transgenic plants having altered saturate levels is the expression of multiple chimeric genes at once. European Patent Application 0400246A1 describes transformation of two *Bt* genes in a plant; however, these could be any two genes or fragments thereof in either the sense or antisense orientation. For example, commercially available hybrids have now been produced having stacked traits such as herbicide and insect resistance. The options could include but are not limited to genes and fragments encoding the palmitoyl-CoA Δ-9 desaturase from *Aspergillus nidulans* with acyl-ACP thioesterase genes or genes encoding proteins such as stearoyl-ACP desaturase, β-ketoacyl synthase II and the like, as well as genes to impart insect control or herbicide resistance. Another way to produce a transgenic plant having multiple traits is to produce two plants, with each plant containing the oil modifying gene of interest. These plants can then be back-crossed using traditional plant breeding techniques available and well-known to those skilled in the art to produce plants wherein phenotypic characteristics are related to the presence of more than one chimeric gene.

The particular embodiments of this invention are further exemplified in the Examples. However, those skilled in the art will readily appreciate that the specific experiments detailed are only illustrative of the invention as described more fully in the claims which follow thereafter.

EXAMPLE 1

ISOLATION AND CLONING OF A FUNGAL PALMITOYL-CoA Δ-9 DESATURASE

Total RNA was isolated from 1.1 g of fresh *Aspergillus nidulans* cells by freezing and grinding said cells in a mortar and pestle which were pre-chilled with

liquid N_2. Before grinding, a small amount of glass beads (150-212μM; SIGMA Chemical Company, St. Louis, MO) was added. The resulting powder was transferred to a centrifuge tube containing 10 mL liquidified phenol equilibrated with 0.1 M Tris-HCl, pH 8.0 and vortexed for 1 min. Organic and aqueous phases were separated by centrifugation at 4° C and the aqueous phase was transferred to a fresh tube, extracted three times with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and once with chloroform/isoamyl alcohol (24:1 v/v). Nucleic acids were precipitated by adding 0.8 volume isopropanol, incubated at -20° C for 1 h, followed by collection by centrifugation. The resulting pellet was resuspended in 5 mL DEPC-H_2O (H_2O having 0.1% v/v diethylpyrocarbonate). RNA was precipitated by adding 3 mL of 8.0 M LiCl followed by incubation on ice for 1 h. Precipitates were collected by centrifugation, resuspended in 5 mL DEPC-H_2O and LiCl precipitated again. The final RNA pellet was resuspended in 500 μL DEPC-H_2O and yield was determined by A_{260nm}. RNA purity and quality was confirmed by electrophoresis on agarose gel.

PolyA$^+$ RNA was purified on oligo dT-cellulose (Collaborative Biomedical Products, Bedford, MA) columns. Type 3 oligo-dT cellulose (0.1 g) was equilibrated in 5 mL of buffer 1 for 30 min, wherein buffer 1 was loading buffer with 0.5 M NaCl and loading buffer was 20 mM Tris-HCl, pH 7.6, 1 mM ethylenediaminetetraacetic acid (EDTA), and 0.1% sodium lauryl sulfate (SDS). The poured column was washed with 3 volumes of DEPC-H_2O, 3 volumes of wash buffer [0.1 N NaOH, 5 mM EDTA], 3 volumes of DEPC-H_2O, and 5 volumes of buffer 1. One mg of *Aspergillus nidulins* total RNA was heated at 65° C for 5 min, diluted 2x with buffer 2 [2x loading buffer] and then applied to the

oligo-dT column. The flow through material was collected, reheated, and reapplied to the column. The column was then washed with 10 volumes of buffer 1 followed by 10 volumes of buffer 3 [loading buffer having 0.1 M NaCl]. PolyA$^+$ RNA was eluted with 3 volumes of elution buffer [10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.05% SDS] and collected in 0.5 mL fractions. RNA fractions were combined, buffered to 0.3 M sodium acetate pH 5.2, and precipitated at -20° C for 16 h after addition of 2.2 volumes of 100% ethanol. The precipitate was collected by centrifugation, washed with 70% ethanol, dried, and dissolved in 50 µL DEPC-H_2O. This material was then repurified on a fresh oligo-dT column as described herein to produce highly-enriched polyA$^+$ mRNA.

Three µg polyA$^+$ mRNA was converted to cDNA and cloned into the LAMBDA UNI-ZAP vector, using the LAMBDA ZAP cDNA synthesis and cloning kit according to the manufacturers protocols (Stratagene, La Jolla, CA). The library had an original titer of 7.0×10^5 pfu/mL. The library was amplified according to Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Laboratory Press) and had a titer of 3.5×10^{10} pfu/mL. The quality of the library was determined by analysis of individual clones. Clones had inserts ranging in size from 0.85 to 1.6 kb.

Total library cDNA was batch rescued and isolated as follows: 5 mL of XL1 Blue MRF' *E. coli* cells (Stratagene), at OD_{600nm} = 1.0 in 10 mM MgSO4, were mixed with 1 µL (3.5×10^7 pfu) of amplified library phage stock, 10 µL (1.0×10^8 pfu) ExAssist helper phage (Stratagene), and incubated at 37° C for 15 min. The mixture was added to 20 mL Luria-Bertani (LB) broth [10 g/L Tryptone, 5 g/L yeast extract,

10 g/L NaCl] and incubated at 37° C for 3.5 h. The cells were heat killed by incubation at 68° C for 0.5 h and cell debris was removed by centrifugation. One hundred µL of *E. coli* SOLR cells (Stratagene) at OD_{600nm} = 1.0 in 10 mM $MgSO_4$ were mixed with 1.0 mL supernatant and incubated at 37° C for 15 min. The mixture was used to inoculate 100 mL of Terrific Broth (TB) [12 g/L Tryptone, 24 g/L yeast extract, 4 mL/L glycerol, 17 mM KH_2PO_4, 72 mM K_2HPO_4] containing Ampicillin at 100 µg/mL. After overnight growth at 37° C plasmid DNA was prepared using alkaline lysis/CsCl purification according to Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Laboratory Press). Yield of batch rescued cDNA was determined by A_{260nm}.

To isolate a clone encoding an *Aspergillus* palmitoyl-CoA Δ-9 desaturase, a DNA fragment was amplified using polymerase chain reaction technology, hereinafter PCR, to produce a probe which could be used to isolate a full length cDNA. A 5' primer and a 3' primer entered herein as SEQ ID NO:1 and SEQ ID NO:2, respectively, were synthesized on an Applied Biosystems High-Throughput DNA Synthesizer Model 394 (Foster City, CA). Batch-rescued maize embryo cDNA was used as template. PCR amplification was performed as follows: 200 ng template DNA, 10 µL 10x reaction buffer, hereinafter 10X RB, [100 mM Tris.HCl pH 8.3, 500 mM KCl, 15 mM MgCl2, 0.01% (w/v) gelatin], 10 µL of 2 mM deoxyribose nucleotides triphosphate (dNTPs), 3000 pmol primers (SEQ ID NO:1 and SEQ ID NO:2), 2.5 units AMPLITAQ DNA Polymerase (Perkin-Elmer, Norwalk, CT) and H_2O for a total volumne of 100 µL. A DNA Thermal Cycler (Perkin-Elmer Cetus Model #480) was programmed as follows: 96° C for 1 min; [94° C (30 sec), 37° C (30 sec), 72° C (2

min)] x 40 cycles; followed by a 7 min (72° C) extension. A DNA product of 119 bp was obtained, sequenced as described below, and entered herein as SEQ ID NO:3. The DNA (SEQ ID NO:3) was cloned into the pCRI1 vector (Invitrogen, Carlsbad, CA) after gel purification on a 1% preparative SEAKEM GTG agarose gel (FMC, Rockland, ME) in TAE [0.04 M Tris-acetate pH 8.1, 0.002 M EDTA]. DNA was extracted from agarose using GenElute Agarose Spin Columns (Supelco Inc., Bellefonte, PA) according to the manufacturer. Ligations and transformations were performed using the Original TA Cloning Kit (Invitrogen). Transformations were plated on LB-agar plates containing 25 μg/mL kanamycin and 50 μg/mL 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside, hereinafter X-gal and allowed to grow overnight at 37° C. White colonies were isolated and grown in 2 mL of LB broth with 25 μg/mL kanamycin and plasmid DNA was extracted using alkaline lysis minipreps according to Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Laboratory Press). Plasmids containing the gene of interest were selected using restriction digest with EcoR1 to screen for an insert of about 120 bp.

Recombinant clones were sequenced by dideoxy chain termination using PRISM AMPLITAQ READY REACTION DYEDEOXY Terminator cycle sequencing kit #401384 according to the manufacturer (Perkin-Elmer Applied Biosystems Division, Foster City, CA). Samples were run on an ABI373A automated DNA sequencer (Perkin-Elmer, Applied Biosystems Division). DNA sequence analysis of SEQ ID NO:3 was performed using MACVECTOR v. 4.1.4 (Oxford Molecular, Campbell, KY), which gave theoretical translation thus generating the amino acid sequence entered herein as SEQ ID NO:4. The first six and last six amino acids of SEQ ID

NO:4 correspond to the translational products of the PCR primers SEQ ID NO:1 and SEQ ID NO:2. The remaining amino acid sequence corresponded to a putative partial desaturase sequence from *Aspergillus nidulans*.

The DNA fragment corresponding to SEQ ID NO:3 was cut out of the vector by digestion with EcoR1 and purified using GenElute Agarose Spin Columns (Supelco). An [α^{32}P]-deoxyribocytosine triphosphate (dCTP)-labeled probe was generated using HIGHPRIME Random Labeling kit (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer using 5 μL of [α^{32}P]-dCTP (3000 Ci/mmole, 10 μCi/μL, DuPont, NEN Life Science Products, Boston, MA). The labeled probe was purified over NucTrap push columns (Stratagene) according to the manufacturer's procedures. Methods for phage titering, plating, coring and rescuing were performed in the LAMBDA ZAP II Library (Stratagene) instruction manual and were used herein. The cDNA library described herein was plated (50,000 pfu/plate) on four 24.3 x 24.3 cm NUNC assay plates (Nunc Inc. Roskilde, Denmark). Duplicate phage lifts were taken from each plate using 0.45 μm MAGNAGRAPH-NT nylon membrane (MSI, Westborough, MA). Filters were treated as follows: 5 min with 0.5 N NaOH/1.5 M NaCl, pH 12.8; 5 min air dry; 5 min with 0.5 M Tris, pH 7.6/1.5 M NaCl; and 5 min air dry. DNA was cross-linked to the membranes while on filter paper dampened with 2x SSC [1x SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0] using a STRATALINKER UV Crosslinker (Stratagene). Filter prehybridization was performed at 42° C in 150 mL hybridization buffer containing 50% (v/v) formamide, 6x SSC, 10x Denhardt's solution [1x Denhardt's solution is 0.02% Ficoll (Type 400, Pharmacia), 0.02% polyvinylpyrollidone, and 0.02% bovine serum albumin], 0.1% (w/v) SDS, and 200 μg/mL

sheared and denatured salmon sperm DNA. After 3 h, used hybridization buffer was replaced with 100 mL of fresh hybridization buffer containing labeled probe having a specific activity = 5 x 10^8 dpm/μg. Hybridization continued for 18-20 h at 42° C with gentle rotation. Afterwards, filters were washed twice at 55-60° C for 40 min in 1 L of wash solution containing 0.2x SSC and 0.1% SDS. Filters were then exposed to Kodak XOMAT-AR Film (Eastman Kodak Company, Rochester, NY) with intensifying screens (Lightening Plus, DuPont CRONEX, DuPont, Wilmington DE) for 16 h at -70° C. Examination of films allowed the identification of positive plaques. Positive plaques were cored out and stored in 1 mL SM buffer [5.8 g/L NaCl, 2 g/L MgSO4, 20 mM Tris.HCl, pH 7.5, 5 mL/L of 2% (w/v) gelatin] with 50 μL chloroform. Phage were plated for secondary screening using 50 μL of a 1:1000 dilution of the primary phage stock. Positive plaques from the secondary screening were cored out and stored in 500 μL of SM buffer. Positive phage were then plated for tertiary screenings using amounts ranging from 5 μL of undiluted secondary stock to 20 μL of 1:100 dilution in SM buffer. All subsequent hybridizations were performed as described above. Isolates were rescued into phagemid form according to the LAMBDA-ZAP II Library Instruction Manual (Stratagene). Rescued phagemid were plated by combining 200 μL SOLR cells (Stratagene) grown to OD_{600nm} = 0.5 to 1.0 with 50 to 100 μL phagemid and incubating for 15 min at 37° C. Cells containing phagemid were streaked on LB agar containing Ampicillin (75 μg/mL) and grown overnight at 37° C. DNA was extracted from 2 mL liquid cultures grown overnight at 37° C in LB medium containing 100 μg/mL ampicillin. DNA was isolated by alkaline lysis minipreps, digested with EcoR1 and Xho1, and fractionated on 1.0%

agarose gels. The DNA was transferred from the gel to Hybond N nylon membrane (Amersham Corporation, Arlington Heights, IL). Clones containing inserts with homology to the 119 bp desaturase probe were identified by hybridization using the ECL direct nucleic acid labeling and detection system (Amersham) according to the manufacturer's instructions. Clones hybridizing with the probe had inserts ranging in size from 0.7 to 1.6 kb.

Miniprep DNA from the positive clones was transformed into *E. coli* DH5α (Gibco-BRL Life Technologies, Bethesda, MD), streaked for single colonies, and plasmid DNA was prepared by the alkaline lysis/CsCl procedure according to Sambrook et al. (Molecular Cloning, A Laboratory Manual, 2nd Ed. (1989), Cold Spring Harbor Laboratory Press). Plasmid was sequenced with primers located in the vector flanking the insert and primers based on the sequence of the internal PCR fragment. A second round of primers was designed based on the sequence obtained from the first round of sequencing. Sequencing was performed as described, *supra.*, compiled, aligned and edited with the SEQED program (Perkin-Elmer, Applied Biosystems Division). The resulting DNA sequence was entered herein as SEQ ID NO:5. DNA sequence analysis of SEQ ID NO:5 was performed using MACVECTOR v. 4.1.4 (Oxford Molecular, Campbell, KY), which gave theoretical translation thus generating the amino acid sequence entered herein as SEQ ID NO:6.

EXAMPLE 2
CONSTRUCTION OF PLANT TRANSFORMATION VECTORS

In order to express the *Aspergillus* desaturase in maize in a constitutive manner, the open reading encoded by SEQ ID NO:5 was cloned in plasmid pDAB439 between the ubiquitin promoter/intron and Nos terminator, thus making pDAB463. Plasmid pDAB439 was a 7040 base pairs double

stranded plant transformation vector composed of the following sequences in clockwise order. The plasmid backbone was derived from pUC19 (Yanish-Perron et al., (1985) Gene 33:103-119). Nucleotides 1 to 2252 of pDAB439 corresponded to the reverse complement of nucleotides 435 to 2686 of pUC19. Nucleotides 2253 to 2271 of pDAB439 had the sequence TGCATGTGTT CTCCTTTTT. Nucleotides 2272 to 4264 of pDAB439 were the maize ubiquitin promoter and first intron, and were PCR amplified from genomic DNA of maize genotype B73 (Christensen et al., (1992) Plant Mol. Biol. 18:675-689). Nucleotides 4265 to 4308 of pDAB439 had the sequence GGTACGGCCA TATTGGCCGA GCTCGGCCTC TCTGGCCGAT CCCC. Nucleotides 4309 to 4576 of pDAB439 corresponded to nucleotides 4420 to 4687 of plasmid pBI101 (Clontech, Palo Alto, CA) followed by the linker GG as nucleotides 4577 and 4578 of pDAB439. Nucleotides 4579 to 4743 of pDAB439 were the reverse complement of nucleotides 238-402 of pUC19. Nucleotides 4744 to 4807 of pDAB439 corresponded to: GCGGCCGCTT TAACGCCCGG GCATTTAAAT GGCGCGCCGC GATCGCTTGC AGATCTGCAT GGG. Nucleotides 4808-5416 of pDAB439 comprised the double enhanced 35S promoter, with nucleotides 5070 to 5416 corresponding to nucleotides 7093 to 7439 of the Cauliflower Mosaic Virus genome (Franck et al., (1980) Cell 21:285-294). Nucleotides 4808 to 5061 of pDAB439 were a duplication of nucleotides 5068 to 5321. Nucleotides 5062 to 5067 of pDAB439 comprised the linker CATCGA. Nucleotides 5417-5436 of pDAB439 comprised the linker GGGGACTCTA GAGGATCCAG. Nucleotides 5437 to 5547 of pDAB439 corresponded to nucleotides 167 to 277 of the Maize Streak Virus genome (Mullineaux et al., (1984) EMBO J. 3:3063-3068). Nucleotides 5548 to 5764 of pDAB439 corresponded to the modified first intron of the maize

alcohol dehydrogenase gene (Adh1-S) (Dennis et al., (1984) Nucleic Acids Res. 12:3983-4000). The modification resulted in removal of 343 nucleotides (bases 1313 to 1656) with bases 1222 to 1312 (intron 5' end) and nucleotides 1657 to 1775 (intron 3' end) of the maize Adh1-S gene remaining. Nucleotides 5765 to 5802 of pDAB439 corresponded to Maize Streak Virus (MSV) nucleotides 278 to 312, followed by the linker sequence CAG. Both sections of the Maize Streak Virus, hereinafter MSV, sequence comprised the untranslated leader of the MSV coat protein V2 gene, and were interrupted in plasmid pDAB439 by the modified Adh1 intron. Nucleotides 5803 to 6359 of plasmid pDAB439 corresponded to nucleotides 29 to 585 of the phosphinotricin acetyl transferase (BAR) gene of Streptomyces hygroscopicus (White et al., (1989) Nucleic Acids Res. 18:1062). To facilitate cloning, nucleotides 34 and 575 of the published sequence were changed from A and G to G and A, respectively. This sequence served as the selectable marker in plant cells. Nucleotides 6360 to 6364 comprised the linker GATCT. Nucleotides 6365 to 6635 of pDAB439 corresponded to nucleotides 4420 to 4683 of plasmid pBI101 (Clontech, Palo Alto, CA) followed by the linker sequence AGATCGC. Nucleotides 6636 to 6639 of pDAB439 comprised the linker TCGG. The remaining sequence of pDAB439 (nucleotides 6640 to 7040) corresponded to nucleotides 284 to 684 of pUC19.

SEQ ID NO:5 was modified so that it could be placed into plasmid pDAB439. To this end SEQ ID NO:5 was amplified with primers as disclosed herein in SEQ ID NO:7 and SEQ ID NO:8. Amplification was performed in six simultaneous reactions as follows: 200 ng template DNA (SEQ ID NO:5), 10 μL 10x RB, 10 μL of 2 mM dNTPs, 3000 pmol primers (SEQ ID NO:7 and SEQ ID NO:8), 2.5 units

AMPLITAQ DNA Polymerase (Perkin-Elmer, Norwalk, CT) and water (total volume = 100 μL). A DNA Thermal Cycler (Perkin-Elmer Cetus Model #480) was programmed as follows: 96°C for 1 min; [94°C (30 sec), 72°C (2 min)] for 15 cycles; followed by a 7 min (72°C) extension. Following amplification, reactions were pooled, the DNA was precipitated with ethanol, and the pellet was resuspended in 40 μL TE buffer [10 mM Tris.HCl pH 8.0, 1 mM EDTA]. Twenty μL DNA was digested with 60 units Sfi1 in 60 μL volume, electrophoresed on a preparative 1% agarose gel, and the liberated 1.4 kbp fragment was isolated from the gel using GenElute columns. The purified DNA was ethanol precipitated and the pellet was resuspended in 20 μL TE buffer. Two μL of fragment were ligated into 100 ng pDAB439 which had been digested with Sfi1. Ligations, transformation, and analysis of recombinant clones was done according Sambrook et al. A clone containing the 1.4 kbp insert was selected and sequenced. The sequence of the insert was identical to nucelotides 4-1371 of SEQ ID NO:5, with exception being the changes that were introduced deliberately, to improve the translation context around the ATG codon. This plasmid was named pDAB463.

In order to express the *Aspergillus* desaturase in maize in a seed specific manner, the ubiquitin promoter/intron in pDAB463 were replaced by the promoter of the maize globulin gene. The globulin promoter was amplified from maize genomic DNA and cloned in plasmid pGGN62-2. Plasmid pGGN62-2 was a 6321 base pair plasmid comprised of the following: nucleotides 1 to 1257 corresponded to nucleotides 4 to 1260 of SEQ ID NO:9; nucleotides 1258 to 3399 corresponded to bases 898 to 3039

of pBI221 (Clontech) in which eight bases of the β-glucuronidase gene, hereinafter GUS gene, were reengineered to contain an Nco1 site at the ATG start codon to facilitate cloning and maintain sequences optimal for translation initiation. This resulted in the first eight base pairs of the GUS gene having the sequence CCATGGTC resulting in an amino acid sequence change from Met Leu to Met Val. The remaining nucleotides in pGGN62-2 (3400 to 6321) corresponded to nucleotides 1 to 2916 of pBLUESCRIPT SK- (Stratagene) with nucleotide 1 being defined as the first A residue of the unique Hind3 site and proceeding clockwise towards the Xho1 site. The six base difference in the number of bases was due to base deletions in the published sequence from 232 to 235 and 663 to 664.

In order to subclone the globulin promoter into plasmid pDAB463, a unique Pac1 site was created upstream of the globulin promoter in plasmid pGGN62-2. An Xba1 to Pac1 adapter having the sequence CTAGCTTAAT TAAG was phosphorylated with ATP and T4 polynucleotide kinase, annealed and ligated into pGGN62-2 which had been digested with Xba1 and treated with Calf Intestinal Phosphatase according to Sambrook et al. Clones containing the adapter were screened by digestion of minipreps with Pac1, and one clone which was cut by Pac1 and not by Xba1 was named pGGN62-2P1. The globulin promoter fragment was cut out by digestion with Pac1 and Nco1, purified by preparative gel electrophoresis, and GenElute columns. Plasmid pDAB463 was cut to completion with Pac1, and a partial digestion was performed with Nco1. The linear fragment of 6.4 kbp was purified by preparative gel electrophoresis and GenElute columns, and following ethanol precipitation was ligated to the globulin promoter fragment. Clones having

the globulin promoter upstream of the *Aspergillus* desaturase were screened by digestion of miniprep DNAs with NcoI. One plasmid having the correct digestion pattern, was named pDAB470 and was sequenced across the cloning junction to verify that sequences around the ATG codon had not been altered.

The *Aspergillus* desaturase was also be used to modify lipid composition of dicot species. In order to express the gene in a seed specific manner, the *Aspergillus* desaturase was placed behind the phaseolin promoter from *Phaseolus vulgaris*. This promoter has been extensively characterized and was shown suitable for high level, seed specific, expression in tobacco. The phaseolin promoter/*Aspergillus* desaturase gene were placed into plasmid phaGN184-2.

Plasmid phaGN184-2 was constructed as follows. The maize expression vector, phaGN184-2, containing the 5' regulatory element from the β-phaseolin gene of *Phaseolus vulgaris* driving the β-glucuronidase gene was used in the expression studies. Plasmid phaGN184-2 was a 6657 base pairs double stranded plant transformation vector composed of the following sequences in clockwise order. Nucleotides 1 to 64 had the polylinker sequence from several subclonings and included the sequence CCACCGCGGT GGCGGCCGCT CTAGATGCAT GCTCGAGCGG CCGCCAGTGT GATGGATATC TGCA. Nucleotides 65 through 1611 contained the 5' regulatory sequences from the β-phaseolin gene of *Phaseolus vulgaris* as disclosed in SEQ ID NO:10. Base 1113 of phaGN184-2 (which corresponded to base 1049 of SEQ ID NO:10) was modified from a C to a T to facilitate subsequent cloning. Nucleotide 1612 of phaGN184-2 was a C. Nucleotides 1613 through 3464 corresponded to nucleotides 2551 to 4402 of plasmid pBI101 (Clontech, Palo

Alto, CA). Bases 1613 to 3418 encoded the β-glucuronidase gene of Jefferson et al. (1987 EMBO J. 6:3901-3907) with bases 1616-1618 modified from TTA to GTC to facilitate cloning and maximize translation initiation. Bases 3465 through 3474 were composed of the linker sequence TGGGGAATTG. Bases 3475 through 3744 of phaGN184-2 were composed of 4414 through 4683 of pBI101 (Clontech, Palo Alto, CA). This sequence was followed by linker ATCGGGAATT corresponding to bases 3745 through 3754. The remaining sequence of phaGN184-2 (nucleotides 3755 to 6657) corresponded to reverse complement of nucleotides from the plasmid backbone which was derived from pUC19 (Yanish-Perron et al., (1985) Gene 33:103-119).

To facilitate transfer of the phaseolin promoter from this plasmid to pDAB463, a unique Xba1 site upstream of the phaseolin promoter was changed into a unique Pac1 site, using the adapter as described above. The resulting plasmid, phaGN184-2P1, was cut with Pac1 and Nco1. The liberated phaseolin promoter fragment was purified from gel and ligated into pDAB463, which had been digested to completion with Pac1, partially digested with Nco1 and purified as described above. The resulting plasmids were screened with Nco1, and two clones were identified which had the appropriate restriction pattern. One of these two clones was named pDAB471, and was sequenced across the phaseolin/desaturase junction to verify that no unintended changes had been made during the modification. The phaseolin/desaturase/nosA gene cassette was transferred to binary vector pDAB1542.

The plasmid pDAB1542 was constructed using standard molecular biology procedures. The 10323 base pair sequence is disclosed herein as SEQ ID NO:11. The starting position was the Hind III site (AAGCTT) which

represented bases 602 to 607 of the T-DNA sequence of pTi-15955 from *Agrobacterium tumefaciens* strain 15955, and which has NCBI Accession Number X00493 J05108 X00282. Nucleotides 1 to 579 of SEQ ID NO:11 represented bases 602 to 1184 of pTi-15955, except that the sequence GTAC, representing nucleotides 622-625 of pTi-15955, had been deleted to destroy a Kpn I recognition site. This sequence section included T-DNA Border A (bases 304 to 327). Nucleotides 580 to 597 of SEQ ID NO:11 were remnants of cloning manipulations. Nucleotides 598 to 2027 of SEQ ID NO:11 were derived from *Escherichia coli* transposon Tn903, and corresponded generally to bases 835 to 2264 of NCBI Accession Number J01839, with the following modifications: base 1467 of J01839 (C) was mutated to T (base 1230 of SEQ ID NO:11) to destroy a Sma I recognition site, and base 1714 of J01839 (C) was mutated to T (base 1477 of SEQ ID NO:11) to destroy a Hind III recognition site. Bases 925 to 1740 of SEQ ID NO:11 were an open reading frame encoding the neomycin phosphotransferase I protein from Tn903. Nucleotides 2028 to 2062 of SEQ ID NO:11 were remnants of cloning manipulations. Bases 2063 to 2080 of SEQ ID NO:11 was derived from *E. coli* transposon Tn5 (NCBI Accession Number U00004 L19385), and represented bases 2519 to 2536 of that sequence (complementary strand). Bases 2081 to 2793 of SEQ ID NO:11 represented nucleotides 21728 to 22440 of pTi-15955 (NCBI Accession Number X00493 J05108 X00282). Bases 2794 to 3772 of SEQ ID NO:11 were Tn5 bases 1540 to 2518 (complementary strand), with the following modifications; base 1532 of Tn5 (G) was mutated to T (base 3764 of SEQ ID NO:11 and base 1536 of Tn5 (C) was mutated to G (base 3768 of SEQ ID NO:11) to create a BamH I site. Bases 2967 to 3761 of SEQ ID NO:11 (complementary strand)

were the open reading frame encoding the neomycin phosphotransferase II protein of Tn5. Nucleotides 3773 to 3784 of SEQ ID NO:11 were remnants of cloning manipulations. Bases 3785 to 4174 of SEQ ID NO:11 were bases 5376 to 5765 of NCBI Accession Number V00141 J02048, and composed the 19S promoter of the CabbS strain of Cauliflower Mosaic Virus. Bases 4175 to 4272 of SEQ ID NO:11 comprised a multiple cloning site for the introduction of heterologous DNA fragments into pDAB1542, and included unique restriction enzyme recognition sites for Bgl II (AGATCT), Asc I (GGCGCGCC), Swa I (ATTTAAAT), Srf I (GCCCGGGC), Pme I (GTTTAAAC), Not I (GCGGCCGC), and Pac I (TTAATTAA). Nucleotides 4273 to 4624 of SEQ ID NO:11 represented bases 13926 to 14277 of pTi-15955 (NCBI Accession Number X00493 J05108 X00282), and included the T-DNA Border B as bases 4407 to 4432, and the overdrive sequence as bases 4445 to 4468. Bases 4625 to 4630 were a Hind III recognition site (AAGCTT), which represents the junction between the modified T-DNA portion of pDAB1542 and the plasmid vector components.

Bases 4631 to 5433 of SEQ ID NO:11 were derived from plasmid pR29 (Morrisson, D. A., M.-C. Trombe, M.-K. Hayden, G. A Waszak, and J.-D. Chen, J. Bacteriol. 159:870-876, 1984); the sequence thereof has not been previously disclosed. They were obtained as part of an 1824 base pair Hind III/Ava I fragment containing the erythromycin resistance determinant from pR29. Bases 5434 to 5828 of SEQ ID NO:11 corresponded to nucleotides 1 to 395 of STRERMAM1 (NCBI Accession Number M20334). Bases 5534 to 6448 of SEQ ID NO:11 corresponded generally to EHERMAM (NCBI Acession Number X81655), with the following exceptions: Bases corresponding to nucleotides 5586, 5927, 5930,and 5931 (all G's) of SEQ ID NO:11 were reported as A

residues in EHERMAM. In addition, bases 5933 (T), 5934 (T), 5935 (C), 5936 (T), and 5938 (C) of SEQ ID NO:11 were A residues in EHERMAM. Nucleotides 5943 to 6448 of SEQ ID NO:11 corresponded to bases 1 to 506 of STRERMAM2 (NCBI Accession Number M20335), with bases 5546 to 6280 of SEQ ID NO:11 comprising an open reading frame encoding a putative adenine methylase protein.

Nucleotides 6448 to 8866 of SEQ ID NO:11 represented nucleotides 15435 to 17853 of plasmid RK2 (NCBI Accession Number L27758), with the following exceptions: The L27758 sequence included an additional T between bases 6573 and 6574 of SEQ ID NO:11, and an additional C between bases 6904 and 6905 of SEQ ID NO:11. Also, bases 6651 (G), 7446 (A), 7461 (A), 7479 (A), and 7494 (T) of SEQ ID NO:11 were found as a C, a C, a C, a G, and a C in L27758. Nucleotides 8861 to 9602 of SEQ ID NO:11 represented bases 50632 to 51373 of L27758, and nucleotides 9614 to 10322 of SEQ ID NO:11 were the complementary strand of bases 12109 to 12817 of L277758, with the following exceptions: bases 9742 (T) and 10024 (C) of SEQ ID NO:11 were both A residues in L27758, and base 10191 (T) of SEQ ID NO:11 was not represented in the RK2 sequence of L27758. Bases 9603 to 9613, and bases 10323 of SEQ ID NO:11 were remnants of cloning manipulations.

Plasmid pDAB1542 was digested to completion with Pac1 and Asc1 and treated with Calf Intestinal Phosphatase. Plasmid pDAB471 was digested with Pac1 and Asc1, and the 3.4 kbp insert was purified by gel electrophoresis and GenElute columns, ethanol precipitated and resuspended in 20 µL TE buffer. Seven µL gel purified fragment was ligated to 200 ng pDAB1542 vector, and transformed into *E. coli* DH5α cells. Resulting colonies were screened for presence of the insert by digestion of miniprep DNAs with

Pac1 and Asc1. One resulting clone having the desired restriction pattern was named pDAB473, and was used in subsequent tobacco transformations.

A control plasmid for tobacco transformation (pDAB1542) which containing a phaseolin/GUS/nosA cassette instead of phaseolin/desaturase/nosA, was constructed as follows. pDAB1542 was digested with Pac1 and Srf1, and treated with Calf Intestinal Phosphatase as described by Sambrook et al. Plasmid phaGN184-2P1 was digested with Pac1 and Pvu2, ethanol precipitated and resuspended in TE buffer. Approximately 1 µg digested phaGN184-2 was shotgun ligated to 200 ng vector and transformed into DH5α *E. coli* cells. Clones with inserts were selected by screening miniprep DNAs with Bgl II. Two clones with a 3.1 kbp Bgl II fragment, diagnostic of the presence of the phaseolin/GUS/nosA gene cassette, were identified. One clone, named pDAB474, was used as a control in subsequent tobacco transformations.

EXAMPLE 3
TRANSFORMATION OF TOBACCO WITH A PHASEOLIN/*ASPERGILLUS* DESATURASE/NOS CONSTRUCT AND CONTROL PLASMIDS

The *E. coli* DH5α strains carrying plasmids pDAB473 and pDAB474, and an *E. coli* strain containing plasmid pRK2013 (Clontech), were grown to log phase in YEP media [10 g/L yeast extract, 10 g/L peptone, 5 g/L sodium chloride] containing 50 µg/L kanamycin. *Agrobacterium tumefaciens* strain EHA101S (deposited in Agricultural Research Service Culture Collection (NRRL) 1815 North University Street, Peoria, Illinois 61604; Deposit number XXX) was grown at 28° C to log phase in YEP medium containing streptomycin at 250 µg/L. The cultures were centrifuged to pellet the cells, and each cell pellet was resuspended in 500 µL LB medium. For pDAB473 mating, 100

μL of *E. coli* DH5α/pDAB473 cell suspension was mixed with 100 μL of *E. coli* containing pRK2013 and 100 μL *Agrobacterium* EHA101S. The mixed suspension was plated on LB-agar plates, and incubated at 28° C for 24 h. The cells were scraped of the plate and resuspended in 1 mL of LB medium, and serially diluted from 10^{-3} to 10^{-6} in sterile water. 100 μL of each dilution was plated on YEP agar plates, containing erythromycin at 100 μg/L and streptomycin at 250 μg/L, and were incubated at 28° C for 2 days, until colonies were clearly visible. Ten colonies from the 10^{-5} dilution were streaked out for single colonies on the same medium twice, to ensure that *Agrobacterium* transconjugants were free from contaminating *E. coli*. For each transconjugant, a 4 mL over night culture was grown in YEP containing erythromycin and streptomycin, and plasmid DNA was prepared using the standard alkaline lysis miniprep procedure. Miniprep DNA was digested with EcoR1, and each transconjugant was shown to contain plasmid DNA having the expected restriction pattern. Conjugation of plasmid pDAB474 into *Agrobacterium* was done as described above for pDAB473.

EXAMPLE 4

EXPRESSION OF *ASPERGILLUS* DELTA-9 IN TOBACCO

Tobacco transformation with *Agrobacterium tumefaciens* was carried out by a method similar to published methods (Horsch et al., 1988 Plant Molecular Biology Manual; Gelvin et al, eds., Kluwer Academic Publishers, Boston, MA). To provide source material for the transformation, tobacco seed (*Nicotiana tabacum* cv. Xanthi) were surface sterilized and planted on the surface of TOB- , which is a hormone-free Murashige and Skoog (MS) medium (Murashige and Skoog, 1962 Plant Physiol. 75:473-497) solidified with agar. Plants were grown for 6-8 weeks in a lighted

incubator room at 28-30° C and leaves were collected sterilely for use in the transformation protocol. Approximately 1 cm^2 pieces were sterilely cut excluding the midrib. Cultures of the *Agrobacterium* strains (EHA101S containing pDAB473 or pDAB474), which had been grown overnight on a rotor at 28° C, were pelleted in a centrifuge and resuspended in sterile MS salts and adjusted OD_{600nm}= 0.7. Leaf pieces were dipped therein for about 30 sec, then blotted dry on sterile paper towels and placed right side up on medium TOB+ (MS medium containing 1 mg/L indole acetic acid and 2.5 mg/L benzyladenine) and incubated in the dark at 28° C. Two days later the leaf pieces were moved to medium TOB+ containing 250 mg/L cefotaxime (Agri-Bio, North Miami, FA) and 100 mg/L kanamycin sulfate (AgriBio) and incubated at 28-30° C in the light. Leaf pieces were moved to fresh TOB+ with cefotaxime and kanamycin twice per week for the first two weeks and once per week thereafter. Leaf pieces which showed re-growth of the *Agrobacterium* strain were moved to medium TOB+ with cefotaxime and kanamycin, plus 100 mg/L vancomycin HCl (Sigma). After four weeks, small plants arising from transformed foci were removed, planted onto medium TOB- containing 250 mg/L cefotaxime and 100 mg/L kanamycin, and grown in a lighted incubator room. After 3-4 weeks these plants had grown to a size sufficient that leaf samples could be analyzed for the presence of the transgene. Afterwards, plants were transplanted into soil in the greenhouse and held under standard greenhouse conditions (30° C, 16 H light) until mature, self-pollinated seed capsules had developed. Oil content of said seed capsules were then analyzed as described herein.

EXAMPLE 5

ANALYSIS OF THE FATTY ACID COMPOSITION OF TOBACCO SEEDS TRANSFORMED WITH ASPERGILLUS PALMITOYL-CoA Δ-9 DESATURASE

The procedure for extraction and esterification of fatty acids from plant tissue was a modification of Browse et. al. ((1986) Anal. Biochem. 152:141-145). One to 20 mg of plant tissue was placed in a test tube. After addition of 1 mL of methanolic-HCL (Supelco, Bellefonte, PA), the tubes were purged with nitrogen gas and sealed. Tubes were then heated at 80° C for 1 h and allowed to cool. Fatty acid methyl esters were removed from the reaction mixture by extraction with hexane, which involved adding 1 mL of hexane and 1 mL of 0.9% (w/v) NaCl followed by vigorous shaking. After centrifugation at 16,000 xg for 5 min the top hexane layer was removed and used for FAME analysis. Analysis was performed by injection of 1 μL of sample on a Hewlett-Packard (Wilmington, DE) Series II model 5890 gas chromatograph equipped with a flame ionization detector and a J&W Scientific (Folsom, CA) DB-23 column. The oven temperature was maintained at 150° C throughout the run (20 min) and the flow of the carrier gas (helium) was 80 cm/sec. Conditions allowed separation of the six fatty acid methyl esters of interest having varying carbon lengths: 16:0, palmityl methyl ester; 16:1, palmitoyl methyl ester; 18:0, stearyl methyl ester; 18:1, oleoyl methyl ester; 18:2, linoleoyl methyl ester; and 18:3, linolenyl methyl ester. Data collection and analysis was performed with a Hewlett-Packard Series II Model 3396 integrator and a PE Nelson (Perkin-Elmer) data collection system. The percentage of each fatty acid methyl ester in the sample was taken directly as indicated by the data collection system. Quantitative amounts of each fatty acid methyl ester were calculated using peak areas of a standard (Matreya, Pleasant Gap, PA) having

known amounts of the five fatty acid methyl esters of interest. The amount determined was used to estimate the percentage of each fatty acid per total fresh weight. Adjustments were not made for loss of fatty acids during the extraction and esterification procedure since recoveries typically ranged from 90 to 100% depending on the original amount of the sample. The presence of plant tissue in the extraction mixture had no effect on the recovery of known quantities of standard.

Transgenic tobacco seeds produced as described herein were analyzed at maturity. From each independent plant three seed cases were harvested. Fatty acid methyl esters were extracted from 20 mg seeds for each sample as described above. The data are summarized in Table 3.

Table 3. Fatty acid composition and percent of lipids per fresh weight of tobacco seeds from plants transformed with pDAB473 (*Aspergillus* desaturase construct) and pDAB474 (control construct).

Line	%16:0	%18:0	%16:1	%18:1	%18:2	%18:3	%FW Lipid
473.6	6.99 ± 0.31	1.33 ± 0.14	4.65 ± 0.21	13.89 ± 0.67	71.28 ± 0.20	0.98 ± 0.07	17.33 ± 1.35
473.8	7.10 ± 0.21	0.83 ± 0.10	4.07 ± 0.07	12.52 ± 0.91	73.80 ± 1.25	0.94 ± 0.01	13.11 ± 2.80
473 avg	8.52 ± 1.15	1.27 ± 0.33	3.01 ± 1.17	13.21 ± 1.69	72.25 ± 2.05	1.03 ± 0.14	16.08 ± 3.95
474 avg	10.09 ± 0.42	2.42 ± 0.27	0.22 ± 0.03	13.17 ± 1.44	72.88 ± 1.20	0.81 ± 0.07	24.04 ± 8.98

Transformation of tobacco with pDAB473, containing the *Aspergillus* palmitoyl-CoA Δ-9 desaturase gene expressed under control of the phaseolin promoter led to dramatic changes in the tobacco seed fatty acid composition, when compared to controls (pDAB474). Palmitoleic acid (16:1Δ9), which is normally present in only minute amounts, accumulated to about 4.0%. As a

result the amount of saturated fatty acids was decreased, and both palmitic acid (16:0) and stearic acid (18:0) were affected.

EXAMPLE 6

PRODUCTION AND REGENERATION OF TRANSGENIC *ASPERGILLUS* Δ-9 DESATURASE MAIZE ISOLATES

Type II callus cultures were initiated from immature zygotic embryos of the genotype "Hi-II." (Armstrong et al, (1991) Maize Cooperation Newsletter, pp.92-93). Embryos were isolated from greenhouse-grown ears from crosses between Hi-II parent A and Hi-II parent B or F2 embryos derived from a self- or sib-pollination of a Hi-II plant. Immature embryos (1.5 to 3.5 mm) were cultured on initiation medium consisting of N6 salts and vitamins (Chu et al, (1978) *The N6 medium and its application to anther culture of cereal crops*. Proc. Symp. Plant Tissue Culture, Peking Press, 43-56) 1.0 mg/L 2,4-D, 25 mM L-proline, 100 mg/L casein hydrolysate, 10 mg/L $AgNO_3$, 2.5 g/L GELRITE, and 20 g/L sucrose, with a pH of 5.8. Selection for Type II callus took place for ca. 2-12 weeks. After four to six weeks callus was subcultured onto maintenance medium (initiation medium in which $AgNO_3$ was omitted and L-proline was reduced to 6 mM).

The plasmids pDAB463 and pDAB470 were transformed into embryogenic callus via helium bombardment. For blasting 140 μg of plasmid DNA was precipitated onto 60 mg of alcohol-rinsed, spherical gold particles (1.5 - 3.0 μm diameter) by adding 74 μL of 2.5 M $CaCl_2 H_20$ and 30 μL of 0.1 M spermidine (free base) to 300 μL of plasmid DNA and H_2O. The solution was immediately vortexed and the DNA-coated gold particles were allowed to settle. The resulting clear supernatant was removed and the gold particles were resuspended in 1 ml of absolute ethanol.

This suspension was diluted with absolute ethanol to obtain 15 mg DNA-coated gold/mL.

Approximately 600 mg of embryogenic callus tissue was spread over the surface of Type II callus maintenance medium as described herein lacking casein hydrolysate and L-proline, but supplemented with 0.2 M sorbitol and 0.2 M mannitol as an osmoticum. Following a 4 h pre-treatment, tissue was transferred to culture dishes containing blasting medium (osmotic media solidified with 20 g/L tissue culture agar (JRH Biosciences, Lenexa, KS) instead of 7 g/L GELRITE (Schweizerhall, South Plainfield, NJ). Helium blasting accelerated suspended DNA-coated gold particles towards and into the prepared tissue targets. The device used was an earlier prototype of that described in US Patent #5,141,131 which is incorporated herein by reference. Tissues were covered with a stainless steel screen (104 μm openings) and placed under a partial vacuum of 25 inches of Hg in the device chamber. The DNA-coated gold particles were further diluted 1:1 with absolute ethanol prior to blasting and were accelerated at the callus targets four times using a helium pressure of 1500 psi, with each blast delivering 20 μL of the DNA/gold suspension. Immediately post-blasting, tissue was transferred to osmotic media for a 16-24 h recovery period. Afterwards, the tissue was divided into small pieces and transferred to selection medium (maintenance medium lacking casein hydrolysate and L-proline but having 30 mg/L BASTA (Agrevo)). Every four weeks for 3 months, tissue pieces were non-selectively transferred to fresh selection medium. After 7 weeks and up to 22 weeks, callus sectors found proliferating against a background of growth-inhibited tissue were removed and isolated. The resulting BASTA-resistant tissue was subcultured biweekly

onto fresh selection medium. Following gas chromatography/fatty acid methyl ester, hereinafter GC/FAME, analyses, as described herein, positive transgenic lines were identified and transferred to regeneration media.

Regeneration was initiated by transferring callus tissue to cytokinin-based induction medium, which consisted of Murashige and Skoog salts, hereinafter MS salts, and vitamins (Murashige and Skoog, (1962) Physiol. Plant. 15: 473-497) 30 g/L sucrose, 100 mg/L *myo*-inositol, 30 g/L mannitol, 5 mg/L 6-benzylaminopurine, hereinafter BAP, 0.025 mg/L 2,4-D, 30 mg/L BASTA, and 2.5 g/L GELRITE (Schweizerhall) at pH 5.7. The cultures were placed in low light (125 ft-candles) for one week followed by one week in high light (325 ft-candles). Following a two week induction period, tissue was non-selectively transferred to hormone-free regeneration medium, which was identical to the induction medium except that it lacked 2,4-D and BAP, and was kept in high light. Small (1.5-3 cm) plantlets were removed and placed in 150x25 mm culture tubes containing SH medium (SH salts and vitamins (Schenk and Hildebrandt, (1972) Can. J. Bot. 50:199-204), 10 g/L sucrose, 100 mg/L myo-inositol, 5 mL/L FeEDTA, and 2.5 g/L GELRITE (Schweizerhall), pH 5.8). Plantlets were then transferred to 10 cm pots containing approximately 0.1 kg of METRO-MIX 360 (The Scotts Co. Marysville, OH) in the greenhouse as soon as they exhibited growth and developed a sufficient root system. They were grown with a 16 h photoperiod supplemented by a combination of high pressure sodium and metal halide lamps, and were watered as needed with a combination of three independent Peters Excel fertilizer formulations (Grace-Sierra Horticultural Products Company, Milpitas, CA). At the 3-5 leaf stage,

plants were transferred to five gallon pots containing approximately 4 kg METRO-MIX 360.

Primary regenerants were self- or sib-pollinated, or outcrossed to either elite inbreds or transgenic plants after an additional 6 - 10 weeks in the 5 gallon pots. R_1 seed was collected at 40-45 days post-pollination.

EXAMPLE 7

METHOD FOR PRODUCTION OF MAIZE SOMATIC EMBRYOS AND ANALYSIS OF FATTY ACIDS THEREIN

Embryogenic callus material containing the genes of interest was maintained as described herein. Continuous production of somatic embryos, which made up a large portion of embryogenic callus, was performed by transferring the callus tissue every two weeks. While the somatic embryos continued to proliferate, they usually remained in an early stage of embryo development because of the continued presence of 2,4-D in the culture medium. Somatic embryos could be regenerated into plantlets when callus was subjected to the regeneration procedure described herein. During regeneration, somatic embryos formed roots and a shoot, subsequently ceasing development as an embryo.

Somatic embryos were made to develop as seed embryos by growing embryogenic callus on MS medium containing 6% (w/v) sucrose. The callus was grown for 7 days and then somatic embryos were individually transferred to MS medium with 6% sucrose and 10 μM abscisic acid, hereinafter ABA.

Somatic embryos were assayed for fatty acid composition using GC/FAME 3 to 7 days after growth on MS medium containing 6% sucrose and 10 μM ABA. Their fatty acid composition was compared to the fatty acid composition of embryogenic callus and to maize zygotic embryos 12 DAP (Table 4). Fatty acid composition of embryogenic callus differed from that of somatic embryos

in that the callus had higher percentages of 16:0 and 18:3 while having lower percentages of 18:1 and 18:2. In addition, the percentage of lipid by fresh weight for the embryogenic callus was 0.4% compared to the somatic embryos 4.0%. The fatty acid composition of the zygotic embryos and somatic embryos were very similar and their percentage of lipid by fresh weight were nearly identical. These data validated the use of the somatic embryo culture system as an *in vitro* system for testing the effect of certain genes on lipid synthesis in developing embryos of maize.

Somatic embryos transformed with pDAB463 and pDAB470 were produced from embryogenic callus using the methods described herein. Control somatic embryos were produced from untransformed lines having backgrounds identical to that of the transformed lines. For the lines tested, 16:1Δ9 was detected in somatic embryos with the highest level being was about 2.7%. Detection of 16:1Δ9 was rare in the control lines, and when it was detected, the levels were never higher than about 0.2% in a single embryo. Table 5 shows the total fatty acid composition of somatic embryos produced from lines 463-09 and 463-43, in which 16:1Δ9 averaged about 0.4% and about 1.2% respectively.

Table 4. A comparison of the fatty acid composition of embryogenic callus, somatic embryos and zygotic embryos.

Fatty Acid Methyl Ester	Percent Fatty Acid Composition		
	Embryogenic Callus[a]	Somatic Embryo[ab]	Zygotic Embryo[ac]
16:0	19.4 ± 0.9	12.6 ± 0.7	14.5 ± 0.4
18:0	1.1 ± 0.1	1.6 ± 0.8	1.1 ± 0.1
18:1	6.2 ± 2.0	18.2 ± 4.9	18.5 ± 1.0

18:2	55.7 ± 3.1	60.7 ± 5.1	60.2 ± 1.5
18:3	8.8 ± 2.0	1.9 ± 0.3	1.4 ± 0.2

[a] The percentage of lipid by fresh weight of tissue was 0.4 ± 0.1, 4.0 ± 1.1, and 3.9 ± 0.6 for embryogenic callus, somatic embryo, and zygotic embryo, respectively. [b]Somatic embryos were grown on MS medium containing 6% sucrose and 10 mM ABA. [c] Zygotic embryos were tested 12 DAP.

Table 5. Fatty acid composition of somatic embryos produced from transgenic cultures containing pDAB463.

Culture Line	Average Fatty Acid Content Percent of Total Fatty Acids (± SE)						Fatty Acid Content (% of fresh weight)
	16:0	16:1	18:0	18:1	18:2	18:3	
463-09	13 ± 0.9	0.4 ± 0.2	0.5 ± 0.2	16.7 ± 1.9	67.7 ± 2.2	1.5 ± 0.3	4.7 ± 0.7
Control	12.5 ± 0.6	0.0 ± 0.0	1.3 ± 0.4	18.2 ± 4.5	65.8 ± 4.5	1.5 ± 0.2	4.9 ± 0.6
463-43	12.7 ± 0.7	1.2 ± 0.3	0.3 ± 0.1	19.7 ± 3.1	64.3 ± 3.3	1.6 ± 0.5	4.7 ± 1.2
Control	13.8 ± 0.7	0.0 ± 0.0	1.0 ± 0.2	17.2 ± 2.4	65.8 ± 2.7	1.5 ± 0.3	5.5 ± 1.3

Embryogenic callus from lines 463-09 and 463-43 was used to regenerate plants as described herein. The fatty acid methyl ester analysis procedure, as described herein, was performed on leaf tissue from these plants. Table 6 shows the total fatty acid composition of leaf tissue from lines 463-09 and 463-43, in which 16:1Δ9 averaged about 4.8% and about 5.5% respectively. These levels of 16:1Δ9 represent about a 3-fold or greater increase over that normally found in control leaves. The 16:0 level was reduced by 20% compared to the control in line 463-43.

Pollinations were made with plants from lines 463-09 and 463-43, seed were obtained as described herein, and fatty acid methyl ester analysis was performed on a small portion (0.5 to 1.5 mg) of each seed embryo. The average fatty acid composition of seed which contained 16:1Δ9 is shown in Table 7. The 16:1Δ9 content of lines 463-09 and 463-43 both averaged from about 0.7% to about 1.1%. The 18:0 content of both lines was reduced by approximately 50%. The data described herein demonstrate that an increased production of 16:1Δ9 in somatic embryos, leaves and seeds of maize, can be obtained by transformation with a gene construct composed of an *Aspergillus* palmitoyl-CoA Δ9 desaturase gene driven by a ubiquitin promoter.

Table 6. Fatty acid composition of leaves from plants produced from transgenic cultures containing pDAB463.

Plant Line	Average Fatty Acid Content Percent of Total Fatty Acids (± SE)						Fatty Acid Content (% of Fresh weight)
	16:0	16:1	18:0	18:1	18:2	18:3	
463-09	19.2 ± 1.8	4.8 ± 0.3	0.7 ± 0.1	1.6 ± 0.8	17.0 ± 2.0	45.2 ± 5.1	0.5 ± 0.0
463-43	14.6 ± 2.1	5.5 ± 0.7	0.4 ± 0.1	2.0 ± 0.8	17.8 ± 3.8	50.6 ± 6.2	0.7 ± 0.2
Control	18.3 ± 1.7	1.6 ± 0.7	2.0 ± 0.7	1.5 ± 0.5	17.8 ± 2.9	48.1 ± 7.2	0.8 ± 0.2

Table 7. The fatty acid composition of seed embryos from 463-09 and 463-43.

Plant Line	Average Fatty Acid Content Percent of Total Fatty Acids (± SE)						Fatty Acid Content (% of fresh weight)
	16:0	16:1	18:0	18:1	18:2	18:3	
463-09	15.1 ± 1.2	0.7 ± 0.2	0.8 ± 0.2	23.6 ± 2.6	58.1 ± 2.7	1.1 ± 0.3	21.1 ± 5.5
463-43	15.3 ± 0.9	0.7 ± 0.1	0.7 ± 0.0	26.1 ± 1.1	56.1 ± 0.6	0.7 ± 0.3	34.8 ± 8.3
Control	13.7 ± 0.5	0.0 ± 0.0	1.8 ± 0.2	26.5 ± 1.2	56.5 ± 1.0	0.7 ± 0.1	46.7 ± 5.4

Fatty acid methyl ester analysis of embryogenic callus transformed with pDAB470 showed that 16:1Δ9 was detected in somatic embryos and reached levels of abour 1.8%. Detection of 16:1Δ9 was rare in the control lines, and when it was detected, the levels were never higher than about 0.2% in a single embryo. Table 8 shows the total fatty acid composition of somatic embryos produced from lines 470-10 and 470-12, in which 16:1Δ9 averaged about 0.5% and about 0.4% respectively.

Embryogenic callus from lines 470-10 and 470-12 was used to regenerate plants as described herein. The fatty acid methyl ester analysis procedure, as described herein, was performed on leaf tissue from these plants. The 16:1Δ9 levels in leaves from these plants were normal, as would be expected because of lack of expression of the embryo-specific promoter in leaf tissue. Pollinations were made with plants from lines 470-10 and 463-43, seed were obtained as described herein, and fatty acid methyl ester analysis was performed on a small portion (0.5 to 1.5 mg) of each seed embryo.

Table 8. Fatty acid composition of somatic embryos produced from transgenic cultures containing pDAB470.

Culture Line	Average Fatty Acid Content Percent of Total Fatty Acids (± SE)						Fatty Acid Content (% Of Total)
	16:0	16:1	18:0	18:1	18:2	18:3	
470-10	12.6 ± 1.1	0.5 ± 0.1	0.5 ± 0.1	17.3 ± 2.4	67.1 ± 3.2	1.5 ± 0.4	5.4 ± 1.3
470-12	11.0 ± 0.8	0.4 ± 0.3	0.7 ± 0.2	17.1 ± 2.4	68.8 ± 2.3	1.3 ± 0.3	5.8 ± 0.8
Control	11.9 ± 0.5	0.0 ± 0.0	0.9 ± 0.2	15.3 ± 2.0	70.4 ± 2.1	1.2 ± 0.2	5.0 ± 0.9

The average fatty acid composition of seed which contained 16:1Δ9 is shown in Table 9. The 16:1Δ9 content of lines 470-10 and 470-12 averaged about 0.9% and about 1.7% respectively. The 18:0 content of both lines was reduced by more than about 50%. The 16:1Δ9 content observed in some seed embryo lines was about 3.2%. A reduction in 16:0 content of about 6% and a reduction in total saturated fatty acids of about 10% was observed in both lines. The data described herein demonstrate that an increased production of 16:1Δ9 and a concomitant decrease in 16:0 and total saturated fatty acids in seeds of maize, can be obtained by transformation with a gene construct composed of an *Aspergillus* Δ9 gene driven by an embryo-specific globulin promoter.

Table 9. The fatty acid composition of seed embryos from 470-10 and 470-12.

Plant Line	Average Fatty Acid Content Percent of Total Fatty Acids (± SE)						Fatty Acid Content (% of Fresh Weight)
	16:0	16:1	18:0	18:1	18:2	18:3	
470-10	10.9 ± 0.6	0.9 ± 0.4	0.5 ± 0.1	18.6 ± 1.1	68.6 ± 1.3	0.4 ± 0.1	25.7 ± 8.9
470-12	10.6 ± 0.4	1.7 ± 0.4	0.4 ± 0.1	19.1 ± 1.2	67.1 ± 1.4	0.4 ± 0.1	27.9 ± 6.6
Control	11.9 ± 0.8	0.0 ± 0.0	1.4 ± 0.2	16.3 ± 1.4	69.3 ±1.21	0.7 ± 0.1	30.0 ± 4.5

EXAMPLE 8

IDENTIFICATION OF 16:1Δ9 IN AN EXTRACT FROM A SEED EMBRYO TRANSFORMED WITH *ASPERGILLUS* Δ-9 DESATURASE

Fatty acid methyl esters were extracted from a seed embryo produced from line 470-12 as described herein. The 16:1Δ9 methyl ester in the extract was identified by comparison of retention time to that of a standard 16:1Δ9 (Matreya, Pleasant Gap, PA). For a typical GC run, the standard 16:1Δ9 and the suspected 16:1Δ9 from the seed embryo extract both had retention times of about 4.3 min.

Further confirmation of 16:1 production involved identification of the suspected 16:1Δ9 peak by gas chromatography-mass spectrometry (GC-MS) and electron impact ionization using a DB-WAX capillary column (J&W Scientific, Folsom, CA) on a Hewlett Packard (Wilmington, DE) 5890 Series II gas chromatograph equipped with a Hewlett Packard 5972 mass selective detector. Initially, the standard 16:1Δ9 was examined to determine the mass spectral fragmentation pattern. This peak eluded at 14.12 minutes and had a mass spectrum with the molecular ion at m/z 268 and fragment ions at m/z 152, m/z 194 and m/z 236. To determine position of unsaturation, an iodine catalyzed dimethyl disulfide derivatization, after a published method (Yamamoto et. at., 1991 Chemistry and Physics of Lipids 60:39), was performed on the standard 16:1Δ9 for 1 h at 35° C. Following addition of hexane/ether and aqueous $Na_2S_2O_3$, the reaction products were analyzed directly by GC-MS. The resultant derivative eluded at 32.46 minutes. The mass spectrum of this derivatized standard had a molecular ion present at m/z 362 and major fragment ions occurring at m/z 145 and m/z 217. This cleavage pattern between the methylthio-substituted carbons was used to determine the double-bond position as being between the C9

and C10 position relative to the acid portion of the molecule in the 16:1Δ9 standard.

The extract from the 470-12 seed embryo was analyzed by GC-MS. This sample contained the suspected 16:1Δ9 peak at about 14.11 min with a fragmentation pattern consistent with the standard 16:1Δ9 methyl ester (molecular ion at m/z 268 and fragment ions at m/z 152, m/z 194 and m/z 236). After derivatization of this sample as described herein, the peak shifted from about 14.11 min to about 32.43 min. The mass spectrum produced from the approximate 32.43 min peak was consistent with the derivatized standard (molecular ion present at m/z 362 and major fragment ions occurring at m/z 145 and m/z 217). These results indicated that the suspected 16:1Δ9 methyl ester in the 470-12 sample is indeed 16:1Δ9 and that the protein encoded by the gene disclosed herein is truly a palmitoyl-CoA Δ-9 desaturase.

EXAMPLE 9
DESIGNING A GENE ENCODING THE *ASPERGILLUS* DELTA-9 DESATURASE FOR HIGH LEVEL EXPRESSION IN MAIZE

A new DNA sequence is chemically synthesized in such fashion that the amino acid sequence of the protein encoded by the new DNA sequence is substantially the same, or identical, to the *Aspergillus* palmitoyl-CoA Δ-9 desaturase amino acid sequence as set forth in SEQ ID NO:6. As described herein, substitutions are made for the nucleotides of the native gene sequence in such a manner as to conserve the identity of the encoded amino acid. However, alterations in codon composition of the new DNA sequence are made such that the overall codon composition of the new DNA sequence more closely resembles the overall codon composition found in maize genes that encode proteins. Furthermore, the choice of said codons used to

substitute for the native codons is preferably the most abundantly used maize codon, but choices can also be made amongst the less preferred maize codon choices to fulfill such desirable attributes as to increase the number of TG and CT base doublets, to decrease the numbers of CG and TA doublets, to remove intron splice sites, to remove polyadenylation signal sequences, to add or remove restriction enzyme recognition sequences, or to add or remove other sequences which may enhance or detract from, respectively, the overall expression level of the gene, as is understood by those skilled in the art. Such an example of a redesigned gene suitable for high level expression in maize plants is disclosed herein as SEQ ID NO:12. Except for the addition of a new alanine residue encoded by the second codon, the encoded protein of SEQ ID NO:12 is identical to the protein encoded by the native *Aspergillus* delta-9 desaturase gene as disclosed herein As can be seen by examination of Table 10, the native *Aspergillus* delta-9 desaturase gene has a codon composition substantially different from that employed by maize, particularly for the arginine CGT and AGG codons, the serine AGC codon, and the glutamine CAA codon. As is also disclosed in Table 10 the redesigned coding region disclosed as SEQ ID NO:12 employs a codon composition that reflects the average codon composition of maize genes that encode proteins, except that codons that are used less than 10% of time in maize genes are avoided. The redesigned gene has a content of G plus C residues of 56.8%, well within the range of other maize genes that encode proteins.

The gene created as described herein and having the SEQ ID NO:12 can then be cloned into the appropriate vector for expression. As described herein, the maize

codon biased gene (SEQ ID NO:12) can be cloned into pBAD439 and inserted into maize plants as described herein to produce a plant whereby the gene is expressed in a constitutive manner. In addition, the gene can also be cloned 3' to the globulin promoter as described herein for pDAB470 to produce maize plants wherein the maize codon biased *Aspergillus* delta-9 gene is expressed in seed embryos.

TABLE 10. *Aspergillus* codon usage and Maize bias table for creating a maize optimized gene encoding for *Aspergillus* delta-9 desaturase.

Codon	Amino Acid	Number in Native Gene	% Usage in Native Gene	Number in Rebuilt Gene	% Usage in Rebuilt Gene	Maize % Usage
TTT	Phe	1	6.7	4	26.7	24
TTC	Phe	14	93.3	11	73.3	76
TTA	Leu	0	0	0	0	5
TTG	Leu	4	11.4	5	14.3	15
TCT	Ser	6	37.5	2	12.5	14
TCC	Ser	6	37.5	4	25.0	24
TCA	Ser	1	6.3	3	18.8	13
TCG	Ser	2	12.5	3	18.8	16
TAT	Tyr	3	15.8	4	21.1	20
TAC	Tyr	16	84.2	15	78.9	80
TAA	Stop	0	0	0	0	12
TAG	Stop	0	0	0	0	42
TGT	Cys	1	25.0	2	50.0	25
TGC	Cys	3	75.0	2	50.0	75
TGA	Stop	1	100	1	100	46
TGG	Trp	20	100	20	100	100
CTT	Leu	10	28.6	8	22.9	16
CTC	Leu	12	34.3	9	25.7	26
CTA	Leu	1	2.9	3	8.6	10

Table 10. Continued

CTG	Leu	8	22.9	10	28.6	28
CCT	Pro	5	25.0	5	25.0	20
CCC	Pro	8	40.0	4	20.0	25
CCA	Pro	4	20.0	5	25.0	26
CCG	Pro	3	15.0	6	30.0	29
CAT	His	3	15.0	6	30.0	35
CAC	His	17	85.0	14	70.0	65
CAA	Gln	0	0	8	47.1	44
CAG	Gln	17	100	9	52.9	56
CGT	Arg	16	57.1	3	10.7	10
CGC	Arg	11	39.3	10	35.7	34
CGA	Arg	0	0	0	0	4
CGG	Arg	0	0	4	14.3	14
ATT	Ile	8	27.6	9	31.0	27
ATC	Ile	21	72.4	16	55.2	60
ATA	Ile	0	0.0	4	13.8	13
ATG	Met	9	100	9	100	100
ACT	Thr	5	23.8	4	19.0	18
ACC	Thr	11	52.4	9	42.9	45
ACA	Thr	2	9.5	3	14.3	15
ACG	Thr	3	14.3	5	23.8	22
AAT	Asn	0	0	4	23.5	21
AAC	Asn	17	100	13	76.5	79
AAA	Lys	3	12.5	5	20.8	19
AAG	Lys	21	87.5	19	79.2	81
AGT	Ser	0	0	0	0	7
AGC	Ser	1	6.3	4	25.0	26
AGA	Arg	0	0	0	0	8
AGG	Arg	1	3.6	11	39.3	30
GTT	Val	11	31.4	7	20.0	18
GTC	Val	16	45.7	11	31.4	33
GTA	Val	0	0	0	0	7
GTG	Val	8	22.9	17	48.6	42
GCT	Ala	12	28.6	11	25.6	26
GCC	Ala	21	50.0	16	37.2	33
GCA	Ala	3	7.1	6	14.0	15
GCG	Ala	6	14.3	10	23.3	26
GAT	Asp	8	28.6	11	39.3	32
GAC	Asp	20	71.4	17	60.7	68
GAA	Glu	5	31.3	4	25.0	24
GAG	Glu	11	68.8	12	75.0	76
GGT	Gly	22	55.0	10	25	21
GGC	Gly	13	32.5	17	42.5	45
GGA	Gly	3	7.5	5	12.5	13
GGG	Gly	2	5.0	8	20.0	21

Claims

1. An isolated nucleic acid having a nucleotide sequence of SEQ ID NO:5 or SEQ ID NO:12.

2. A protein comprising an amino acid sequence of SEQ ID NO:6.

3. An isolated nucleic acid encoding the protein of SEQ ID NO:6.

4. A DNA construct comprising, in the 5' to 3' direction: a promoter regulatory element, a nucleic acid fragment encoding a palmitoyl-CoA Δ-9 desaturase from *Aspergillus*, and a transcriptional terminator sequence, wherein either said promoter regulatory element or said transcription termination sequence is not naturally associated with said nucleic acid fragment.

5. The construct of Claim 4 wherein said promoter regulatory element is selected from the group consisting of ubiquitin promoter, maize globulin promoter, maize streak virus leader sequence, 35s promoter, 35T promoter, the first intron of maize alcohol dehydrogenase and beta-phaseolin promoter.

6. The construct of Claim 4 wherein said nucleic acid fragment is selected from the group consisting of SEQ ID NO:5 and SEQ ID NO:12.

7. A plant cell containing the nucleic acid construct of any one of claims 4-6.

8. A plant cell of claim 7 wherein said promoter regulatory element is selected from the group consisting of ubiquitin promoter, maize globulin promoter, maize streak virus enhancer region, 35s promoter, doubly enhanced 35s promoter, the first intron of maize alcohol dehydrogenase and beta-phaseolin.

9. A plant cell of Claim 7 wherein said plant cell is selected from the group consisting of soybean,

Brassicaceae sp., canola, rape, sunflower, flax, safflower, coconut, palm, olive, peanut, cotton, castor bean, coriander, *Crambe* sp., *Cuphea* sp., *Euphorbia* sp., *Oenothera* sp., jojoba, *Lesquerella* sp., marigold, *Limnanthes* sp., *Vernonia* sp., *Sinapis alba*, cocoa, tobacco, and maize.

10. A plant cell of Claim 7 wherein said cell is a seed embryo cell.

11. Oil derived from a plant cell of Claim 10.

12. A transgenic plant produced from a plant cell of Claim 7.

13. Seed and progeny thereof produced from a transgenic plant of Claim 12.

14. A method of producing a plant oil having altered levels of fatty acids comprising: growing a plant cell having integrated into its genome a construct comprising, in the 5′ to 3′ direction, a promoter regulatory element functional in a plant cell, a nucleic acid fragment that encodes delta-9 CoA desaturase isolated from Aspergillus and a transcriptional terminator sequence.

15. Plasmid pDAB1542, having the nucleotide sequence of SEQ ID NO:11.

SEQUENCE LISTING

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Merlo, Donald J

<120> Modification of Fatty Acid Composition in Plants by Expression of A Fungal Acyl-CoA Desaturase

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                20                  25                  30

aag cat gtg aac tgg ctg aac gtt ttc ctg atc atc ggt atc ccg ctt   144
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            35                  40                  45

tat ggg tgc att cag gcg ttc tgg gtg cca ctg cag ctg aag act gcc   192
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Ile Trp Ala Val Ile Tyr Tyr Phe Phe Thr Gly Leu Gly Ile Thr Ala
    65                  70                  75

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Gly Tyr His Arg Leu Trp Ala His Cys Ser Tyr Ser Ala Thr Leu Pro
80                  85                  90                  95

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Thr Asp Lys Asp Pro Tyr Ser Val Arg Lys Gly Leu Leu Tyr Ser His
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    225                 230                 235

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            260                 265                 270

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        275                 280                 285

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    290                 295                 300

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Lys Lys Val His Ile Ala Asp Thr Ala Ile Asn Arg His Asn Trp Tyr
14

```
                20                  25                  30

aaa cat gtg aat tgg ctc aat gtg ttc ctg atc atc ggg atc cct ttg   144
Lys His Val Asn Trp Leu Asn Val Phe Leu Ile Ile Gly Ile Pro Leu
          35                  40                  45

tat ggg tgc att caa gcg ttc tgg gtg cca ctc caa ctg aag act gcg   192
Tyr Gly Cys Ile Gln Ala Phe Trp Val Pro Leu Gln Leu Lys Thr Ala
      50                  55                  60

atc tgg gca gtg atc tac tac ttc ttc acc ggt cta ggg atc acg gct   240
Ile Trp Ala Val Ile Tyr Tyr Phe Phe Thr Gly Leu Gly Ile Thr Ala
 65                  70                  75                  80

ggg tat cat agg ctc tgg gct cac tgc tcg tac tcg gca acc cta cct   288
Gly Tyr His Arg Leu Trp Ala His Cys Ser Tyr Ser Ala Thr Leu Pro
                85                  90                  95

ttg agg att tgg cta gct gca gtt ggt gga ggt gca gtc gaa ggc tca   336
Leu Arg Ile Trp Leu Ala Ala Val Gly Gly Gly Ala Val Glu Gly Ser
            100                 105                 110

ata cgc tgg tgg gct cgg gat cac agg gct cac cac cgc tac acc gac   384
Ile Arg Trp Trp Ala Arg Asp His Arg Ala His His Arg Tyr Thr Asp
        115                 120                 125

acc gac aaa gat ccg tac tcc gtt cgg aag ggt ctg ctc tac tct cac   432
Thr Asp Lys Asp Pro Tyr Ser Val Arg Lys Gly Leu Leu Tyr Ser His
    130                 135                 140

ctt ggc tgg atg gtg atg aag cag aac ccg aag cgc att ggc cgc act   480
Leu Gly Trp Met Val Met Lys Gln Asn Pro Lys Arg Ile Gly Arg Thr
145                 150                 155                 160

gac att tcc gac ctc aat gag gat ccc gtg gtt gtc tgg caa cac cgc   528
Asp Ile Ser Asp Leu Asn Glu Asp Pro Val Val Val Trp Gln His Arg
                165                 170                 175

aac tac ctg aag gtg gtg ttc acg atg gga ttg gct gtg ccg atg ctt   576
Asn Tyr Leu Lys Val Val Phe Thr Met Gly Leu Ala Val Pro Met Leu
            180                 185                 190

gtt gct gga ctt gga tgg ggt gac tgg ttg ggc ggc ttt gtg tat gcg   624
Val Ala Gly Leu Gly Trp Gly Asp Trp Leu Gly Gly Phe Val Tyr Ala
        195                 200                 205

ggc atc ctg cgc atc ttc ttc gtc caa cag gcg act ttc tgt gtc aac   672
Gly Ile Leu Arg Ile Phe Phe Val Gln Gln Ala Thr Phe Cys Val Asn
    210                 215                 220

tca ttg gcc cac tgg ctg ggt gac cag ccc ttt gat gac cgc aac tca   720
Ser Leu Ala His Trp Leu Gly Asp Gln Pro Phe Asp Asp Arg Asn Ser
225                 230                 235                 240

cct agg gac cat gtg atc acc gct ctg gtc acc ctt gga gag ggc tac   768
Pro Arg Asp His Val Ile Thr Ala Leu Val Thr Leu Gly Glu Gly Tyr
                245                 250                 255

cac aac ttt cac cat gag ttc ccc tcg gac tac cgc aat gcc att gaa   816
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